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PRINCIPLES OF INSECT PATHOLOGY

BY

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PRINCIPLES OF INSECT PATHOLOGY

To
MABRY

PREFACE

This book was originally intended primarily for the use of students enrolled in the course in insect pathology being given at the University of California. The need of a textbook in this field was a pressing one since not only did the students find the literature on the diseases and other pathological conditions of insects difficult to obtain and to assess, but no reasonably complete treatment of the subject from the student's standpoint was to be had. To fill this need is the first objective of this volume.

Considering the field as a whole, however, it was realized that, in addition to a textbook for classroom use, the subject was also greatly in need of a reference work. The entomologist and the research worker in biology have not had an available guide to all phases of the subject of insect pathology as such. Some aspects of the field have been treated by other authors (such as Paillot's "L'Infection chez les insectes," published in 1933) but not in a general or by any means complete manner. For most of the information the research worker has been forced to consult widely scattered and often almost inaccessible articles or incomplete accounts in larger volumes. In 1946 the author published "Insect Microbiology," purely a reference book, dealing in a general way with the microbiology of insects. Very few details relating specifically to diseases and pathological conditions in insects were included in this previous volume, which was largely an accumulation and summarization of facts relating to the many biological relationships existing between microorganisms and insects. A development of the large subject of insect pathology was not possible within the confines of that publication. Accordingly, the author turned his attention to the preparation of the present book, which deals specifically with insect pathology, *i.e.*, with the microbial diseases of insects as well as with certain amicrobial diseases, injuries, and abnormalities. The task has not been an easy one. The accomplishments of workers in this field frequently are difficult to evaluate and present in a logical, easily readable form. And too, the field of insect pathology is much larger than most persons realize. Furthermore the subject matter is such as to be peculiarly difficult to present both as a textbook and as a reference book. This nevertheless is what I attempted to accomplish.

Although pertinent references have been cited throughout the book, no attempt has been made to make the volume completely bibliographic. The literature contains over 5,000 references dealing with the diseases of

insects, and to attempt to include all these would defeat the purpose of the book. Care has been taken, however, to list those references which will lead the reader to most of the significant contributions made in each particular phase of the subject.

Chapters 2 and 3, dealing with the amicrobic pathologies, have intentionally been held to a minimum, and the emphasis of the book has been placed on the microbial diseases of insects. A substantial portion of Chaps. 2 and 3 may be considered as belonging to the fields of insect toxicology and insect physiology. Nevertheless the pathologies and abnormalities concerned are too frequently ignored by textbook writers in these fields. Since it is important for purposes of orientation that the student in insect pathology be fortified with information dealing with amicrobic pathologies, a brief discussion of the latter is included in the present volume, but the treatment given them is not intended to be comprehensive.

A book of this kind, dealing with several large and distinct groups of microorganisms as well as with insects, is likely to be subject to peccadillos concerning the synonymies and forms of names. An attempt to avoid them has been made by following the nomenclature used by the leading authorities in the various fields concerned. In spite of constant vigilance, errors probably have crept in; and, with regard to the book as a whole, I can but echo Chaucer's humble petition that "if there is anything that displeases them [the readers], I pray also that they ascribe it to the fault of my lack of skill, and not to my intention, which would gladly have expressed it better if I had had the skill to do so."

To write a scientific book it is usually necessary first to have an environment in which such work may be prepared and nurtured. Credit for supplying this environment belongs to Professor Harry S. Smith, of the University of California, who, more than anyone else, made possible not only this volume but the launching of insect pathology as it was initiated and developed at the University of California. As Chairman of the Division of Biological Control, Professor Smith enabled this neglected field (insect pathology) to have its share of academic, technical, and financial attention along with that of parasitic insects and other natural enemies of insect pests. Without the warm encouragement and kindly understanding of Professor Smith, the writing of this book probably would not have been attempted.

I am also greatly indebted to others who have been generous in their help and advice. For critically reading portions of the manuscript, I wish to express my deep appreciation to other colleagues at the University of California, in addition to Professor Smith, especially to Professors Merlin W. Allen, Roderick Craig, and Harold Kirby. I am particularly indebted to Kenneth M. Hughes for reading the entire manuscript and

for preparing certain of the photographs, and to Eunice Crapuchettes and Karl Snyder, who ably prepared most of the line drawings. My thanks also to the numerous persons who kindly provided prints of photographic material and who permitted their reproduction in this book. Acknowledgments of these courtesies are made at the places where the material has been used. I also wish to thank Natalie Ross Herring, who painstakingly typed the manuscript, and who assisted in much of the work of indexing, and Helen Owsley, who assisted in the reading of proof.

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EDWARD A. STEINHAUS

CONTENTS

CHAPTER 1

INTRODUCTION

The term "insect pathology" refers to that branch of entomology which embraces the general principles of pathology as they may be applied to insects. In a broad sense it refers to observations concerning the cause, symptomatology, and epizootiology of the diseases of insects, and to a study of the structural, chemical, and functional alterations in the body of the insect resulting from disease or injury. The words "insect pathology" may be thought of as referring to insects in a manner similar to that in which "plant pathology" refers to plants. From a practical standpoint it may also include certain aspects of the general field of insect microbiology and certain of the biological relationships existing between insects and microorganisms not pathogenic to them. It requires the techniques of, and information from, the sciences of entomology, pathology, bacteriology, mycology, protozoology, virology, and immunology. Insect pathology is nevertheless a separate and distinct branch of entomology, intimately related to each of the other branches of this science.

The field of insect pathology needs no apology for its existence, since it has already made many significant contributions to the fields of general biology, agriculture, and medicine. Of greater concern to most of the readers of this book, however, are the contributions it has made and is making to the various branches of entomology.

Relation of Insect Pathology to Other Branches of Entomology. Perhaps the first apparent application of insect pathology that comes to the mind of the student in entomology is its use in the field of biological control. The biological control of insect pests by means of parasitic insects has been successful in numerous cases in many countries of the world and has saved farmers and growers billions of dollars. It might be expected that the use of microbial control could add to this saving. Since insects are subject to diseases just as are other animals, the possibility of controlling serious insect pests by the dissemination of disease organisms among them not only appears plausible but in several instances has proved to be entirely possible and practicable.

One should not envision the use of microorganisms as the panacea to insect control. Many early reports on the use of fungi and bacteria against insects were overenthusiastic, and some were proved to be ground-

less, lacking the support of controlled experimentation. On the other hand, one should not feel as pessimistic as do some who are willing to abandon the use of microorganisms altogether as a practical means of control. On the basis of our present knowledge of the subject, the proper attitude would seem to be one of hopeful conservatism. At least it should be permissible to express the hope that in a few worth-while instances the artificial dissemination of disease organisms under the proper conditions may aid in at least the partial or seasonal reduction of certain insect populations. In some cases it may be expected that the diseases, once established, may maintain themselves naturally or by reintroduction and take a small but significant toll of insects year after year. In a few instances the microbial control of insects may prove to be so effective and so inexpensive that it may constitute the only practical means of control.

Of even greater significance than the artificial use of these diseases is the very effective control of insects that takes place in nature through the agency of disease without the help of man. The great and tremendous importance of natural microbial control is very little appreciated. Our understanding of these naturally occurring diseases is instrumental to our understanding and use of diseases artificially initiated. We must be willing to spend the time, money, and energy necessary to accomplish the results of fundamental research in this field before we can expect to derive much practical usefulness from it. No longer can substantial progress be made by the hit-and-miss methods of the past. What is urgently needed is the sympathetic, moral, and financial support of basic research into the various biological relationships existing between insects and microorganisms, and into the many factors concerned in the spread of diseases among insects in the field. Other aspects of the subject of the microbial control of insects will be discussed in a later chapter.

Insect pathology may be valuable to economic entomology in ways other than that of artificial biological control. For one thing, the entomologist studying the ecology of insects frequently finds himself depending on an understanding of disease outbreak before he can correctly interpret his other observations. For example, the unexpected decline in Japanese-beetle populations in certain parts of northeastern United States was at first attributed to factors other than the primary one, the presence of the bacterial milky diseases. Had the presence of disease been recognized earlier, it is probable that considerable time and expense, as well as embarrassment, might have been spared. Other examples might be cited, but it seems clear that no ecologist is a good ecologist until he has a thorough understanding of all the principal factors affecting insect populations; and, with the gathering of more information, it is becoming increasingly clear that the diseases and parasites of insects are important

in this regard. Furthermore, it should be of some value to the field entomologist, when he sees insects dying in large numbers, to be able to determine the likely outcome of the outbreak relative to climatic conditions. For example, if he is acquainted with the symptoms of virus diseases, he will know that an extensive epizootic will probably make chemical control unnecessary; if he knows that a fungous disease is concerned, he will realize that weather conditions may have a great deal to do with the progress of the disease; and so on.

Those who rear insects in the laboratory or in insectaries are finding that the control of disease in their insects frequently presents problems for which the field of insect pathology may offer solutions. Sometimes these insectary problems are concerned with chronic diseases, more nuisances than they are explosive outbreaks. On the other hand, a disease may be so serious as to wipe out entire colonies. Anyone who has reared or has attempted to rear lepidopterous insects within cages in the laboratory has probably had the experience of having an outbreak of "wilt disease" completely destroy or ruin his colony or cultures. It is hoped that further study of these problems will make for a solution of them.

Insect physiology and insect pathology have a great deal in common, each contributing greatly to the development of the other. It is now well known that certain of the microorganisms found in insects directly affect the physiological processes of the latter to such an extent that in some cases the life of the insect is absolutely dependent upon the activities of the microorganisms associated with it. Not only do microorganisms influence the ordinary nutritional processes of insects, but in some cases it is definitely known that they supply such substances as vitamins necessary for the insect's growth and development. Diseases of insects always involve a disruption of the metabolism and other physiological processes of an insect, and hence the knowledge gained in the study of these diseases may be in the form of direct contributions to insect physiology. Furthermore, significant contributions have been made to the latter field through the study of the pathological processes concerned in certain of the noninfectious diseases of insects.

The insect morphologist and insect histologist have already received much from the insect microbiologist and pathologist. Some extremely important contributions to insect embryology and histology have been made in conjunction with studies on the intracellular symbiotes found normally in many species of insects. Since many of these microorganisms are passed from generation to generation through the egg, their relationship with the insect host is so intimate that they not only affect the course of the insect's embryological development but in a broad evolutionary sense may be responsible for certain structures, such as mycetomes,

found in insects. A prerequisite to a thorough understanding of the principles of insect pathology is a detailed knowledge of the anatomy and cellular structure of insects. A perusal of numerous papers on diseases of insects reveals many fine contributions on the histology of normal as well as that of diseased tissues. Similarly, insect pathologists have found it necessary in many cases to make careful studies of the blood pictures in diseased and normal insects; these contributions should be added to similar ones being made by the insect physiologist.

Since the full meaning of the term "insect pathology" includes certain aspects of insect toxicology, one need only point out that, when insects are sickened, killed, or otherwise affected by toxic chemical agents, a pathological state results. These pathologies come under the category of non-infectious pathologies or injuries but as such must be studied by techniques and procedures used in insect pathology generally. As will be indicated in the next chapter, this branch of insect pathology—*i.e.*, the study of pathological effects in insect tissues caused by chemical agents—is still largely an open field requiring the skills of the fields of insect toxicology, insect physiology, and insect pathology.

Insect taxonomy and identification, too, has not been without its contributions from insect pathology, and more will undoubtedly be made in the future. For example, races and strains of insects have been separated on a basis of their inherent normal resistance to certain diseases, such as the resistance of certain races of silkworms to the microsporidian disease pebrine. The morphological characteristics of certain of the internal symbiotes of insects have been used to separate species or subspecies. Such use of these microorganisms has, for instance, been claimed in the case of the lac insects of India, which, like most of the soft scales, harbor distinctive internal yeastlike microorganisms more or less characteristic for the species carrying them.

The value of insect pathology to medical entomology in particular, and to parasitology generally, is probably fairly obvious, but it is surprising how little correlation is made between the two. One of the basic maxims of insect pathology is the gaining of an understanding of the biological relationships between insects and microorganisms. This is just the type of information and study so greatly needed in medical entomology. When considered in the large sense, and with certain exceptions, very little is known of the fundamental biological relationships existing between microorganisms affecting man and animals and their arthropod vectors. The same may be said of relationships between microorganisms that cause diseases of plants and their arthropod transmitters. It would appear that a prerequisite to proper understanding of medical entomology and plant-virus transmission is a knowledge of insect microbiology including insect pathology.

Now, just as insect pathology can be useful to other branches of entomology, the converse is true. Insect pathology is absolutely dependent upon every other branch of entomology, excluding none. Not the least of these dependencies is that in which the insect pathologist relies upon the field entomologist to bring him reports of disease outbreaks as well as to send in diseased specimens collected in nature. In the laboratory,

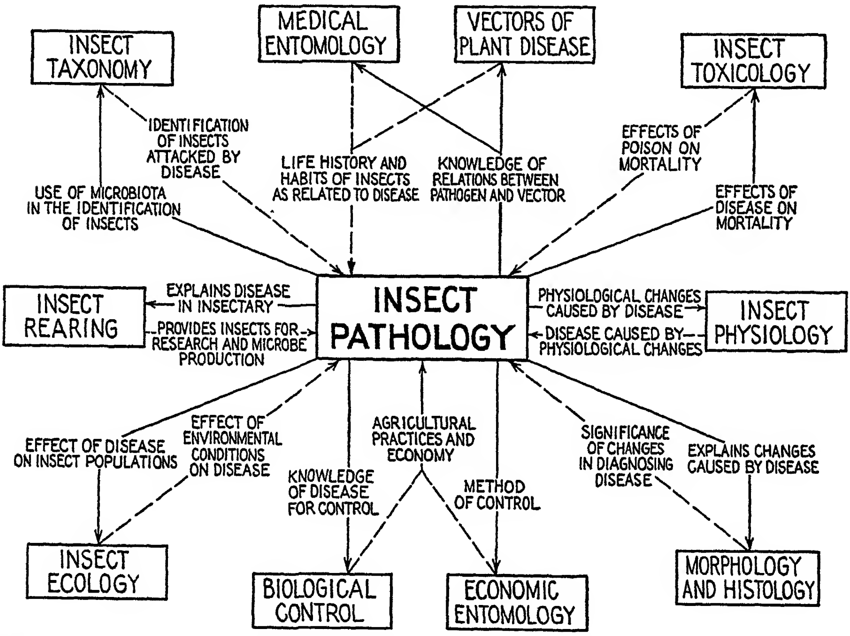

Fig. 1. A diagrammatic representation of the relation of various phases of entomology to insect pathology.

the insect pathologist is dependent upon the techniques and procedures of all the various branches of entomology.

A few of the various relationships existing between insect pathology and other branches of entomology are diagramed in Fig. 1.

Historical Aspects in Brief. Insect pathology may be said to have had its beginnings early in the development of beekeeping and in the establishment of sericulture when certain abnormalities in bees and silkworms were noted by those who reared them for their useful products. For instance, the fact that bees may suffer from disease is recorded in works that were written before the Christian era. It was only about a century ago, however, that the microbial causes of some of these diseases were suspected.

Although as early as 1835 Bassi de Lodi demonstrated that one disease

of the silkworm was caused by a fungus, it remained for the illustrious work of Pasteur to focus the serious attention of scientists on the diseases of insects. Pasteur's first contribution along these lines was that concerning the microsporidian disease pebrine, and in 1870 he published his famous "Etudes sur la maladie des vers à soie," which in addition to pebrine also dealt with the condition in silkworms known as "flacherie." His work unquestionably did much to save the silkworm industry of France at that critical period, and it is not generally realized that the diseases of the silkworm were the first demonstrated microbial diseases of animals. For a more complete account of his work on the diseases of silkworms and how Pasteur became interested in this subject, see Chap. 12.

About this same time, mycologists were beginning to report the parasitization by fungi of insects other than the silkworm. Robin (1853), Fresenius (1856, 1858), Cohn (1855–1869), Bail (1869), Brefeld (1870), and Lohde (1872) were among those making the first scientific observations on entomogenous fungi. Then, in 1879, Metchnikoff, in Russia, made the first significant experiments on the destruction of injurious insects by the use of microorganisms when he was able to infect larvae of the beetle *Anisoplia austriaca* Hbst. with the fungus *Metarrhizium anisopliae* (Metch.). Following this, Krassilstschik, at the University of Odessa, utilized the methods of Metchnikoff against other insects and in 1884 established a special laboratory for the purpose of producing the spores of the fungus on a large scale.

Speculation as to the possible use of fungi in the control of insects was being made in the United States about this time by Hagen (1879) of Harvard University. Also in the United States were such workers as Snow (1890) and Forbes (1895, 1896), who emphasized the use of fungi as a control measure, and Thaxter (1888–1930),[1] who produced classic works on the systematics of various groups of entomogenous fungi, particularly the Entomophthorales and the Laboulbeniales. Other groups of fungi were being given systematic treatment by the English worker Petch. The work of Fawcett, Berger, Watson, and others at the Florida Experiment Station (1908 *et seq.*) brought much attention to the role of fungi in the control of certain insect pests of citrus plants.

Up to 1911 the fungi and protozoa associated with insects were receiving most of the attention, but about this time d'Herelle in Yucatan, Mexico, isolated from dead and dying locusts a bacterium that he called *Coccobacillus acridiorum*. On the basis of d'Herelle's optimistic reports, considerable excitement and great expectations were aroused concerning the use of this bacterium against grasshoppers in various parts of the

[1] For a biographical review of Thaxter's life and work, see the account by Weston (1933).

world. These initial optimistic reports eventually gave way to doubt and discouragement as far as the possibilities of microbial control of insects were concerned.

During this same period, additional observations on diseased insects were being made by isolated workers both in the United States and in Europe. Four men, however, stood out as leaders in the study of the diseases of insects. Beginning in 1904, but with most of his important observations between 1912 and 1936, were the contributions of the American G. F. White, whose additions to our knowledge of the diseases of bees were one of the first generally reliable scientific treatments of this group of infections. Also in the United States was R. W. Glaser, whose contributions began in 1914. It was Glaser who, together with Chapman, introduced American entomologists to the nature of the "wilt disease," or polyhedrosis, of the gypsy-moth caterpillar and other insects. The work of Paillot (1913–1944)[1] in France has proved to be of monumental significance. Paillot's contributions were particularly valuable because many of them dealt with fundamental problems that were so greatly in need of elucidation. He concerned himself with nearly all major phases of insect pathology. Metalnikov and his associates (1914–1935) also worked with fundamental problems, especially those having to do with the immunity principles in insects. The work of both Paillot and Metalnikov has been criticized for its generalizations, but the fact remains that their investigations provided a distinct impetus to the interest in and the development of insect pathology.

During the years just prior to World War II it became increasingly apparent that the field of insect pathology needed not only formalization but also a more definite and direct approach to the solution of its problems. The study of insect diseases as incidental to or as a stepchild of the investigation of other problems was proving itself inadequate. Separate and distinct projects dealing solely with insect pathology were needed, and among the first to take well-planned steps in this direction was the U.S. Department of Agriculture. This agency delegated special experts to the study of the diseases affecting the honeybee and later set up a special unit for the investigation of the milky diseases of the Japanese beetle, a serious pest in eastern United States. In 1945 the College of Agriculture at the University of California established a Laboratory of Insect Pathology, which concerned itself with all phases of insect pathology and through which was offered the first regular formal course in this subject. This laboratory was organized as an integral part of the Division of

[1] For a biographical review of Paillot's life and work see the account in *Compt. Rend. Acad. Sci., Paris,* 1945, **220,** 205–206. A similar account of Glaser's life may be found in *Science,* 1948, **107,** 131–132, and in *J. Parasitol.,* 1948, **34,** 165–168.

G. F. WHITE
(1875–1937)

R. W. GLASER
(1888–1947)

A. PAILLOT
(1885–1944)

S. METALNIKOV
(1870–1945)

Fig. 2. Four pioneer workers noted for their contributions on the diseases of insects. White and Glaser were American workers; Paillot and Metalnikov did their work in France.

Biological Control of the University. By the next year, the Canadian Department of Agriculture had set up facilities through which they undertook to investigate the possible use of virus diseases against certain forest defoliators. All these developments gave the field new emphasis and spoke for a more thorough and a more scientific outlook on all phases of the subject.

More of the historical aspects of insect pathology will be brought out in subsequent discussions of the particular subjects concerned. Some of the landmarks of the development of the field have been mentioned briefly here, principally to assure the reader that insect pathology does have a history of its own even though this fact has often been clouded by associated accomplishments in other fields made at the same time. This state of affairs is highlighted by the fact that the literature in insect pathology is widely scattered in journals covering many different fields. It is a situation that is likely to continue for some time, since insect pathology is still in its infancy, and the field is still in need of further organization as well as in need of greater financial and moral support generally.

TECHNIQUES OF INSECT PATHOLOGY

If Jonathan Swift were writing his "Gulliver's Travels" in this modern day, it is probable that in his land of Brobdingnag many of the "germs" would be large enough to be seen with the naked eye. At any rate, fecal drops of the Brobdingnagian flies would undoubtedly have been even more disturbing to Gulliver if the teeming microbial life they contained had been visible to him. Modern microscopic and cultural techniques quickly take us to the land of Brobdingnag, however, and the Lilliputian inhabitants of insects are made as discernible to us as they might have been to Gulliver.

A knowledge of the use of microscopes and culture methods, although of first importance, is by no means all that is necessary for a proper understanding of the microbiology and pathology of insects. Actually, all the techniques and procedures used in the sciences of entomology, microbiology, immunology, and pathology are of potential use to the insect pathologist. Most of the techniques used in these sciences are well standardized and can be referred to in manuals and textbooks on these subjects. It will be our purpose here simply to indicate very briefly a few of the more important techniques and a general idea as to how they may apply to insect pathology.

Collecting and Shipping Procedures. Probably most of the readers of this volume are familiar with the various procedures and methods used by entomologists in the handling of insects. The insect pathologist, however, may add a few twists of his own in order to ensure the subsequent reliability of results.

Let us suppose that the insect pathologist finds an apparently diseased insect in the field and wishes to make or to have made a thorough diagnosis and pathological examination of the specimen—how does he proceed?

In the first place, it is essential that the specimen be collected and shipped in such a way that it will arrive at the laboratory as nearly as possible in the same condition as it was found in nature. An individual or a small number of individual specimens may be collected in sterile or thoroughly cleaned glass vials. Large numbers of insects may be gathered by means of a collecting net if the cloth has been previously sterilized so as to destroy any infecting organism that may have remained on it from earlier use.

The insects should always be transported to the laboratory by the fastest possible means—by air mail whenever the distance is considerable. For long-distance shipments, as from one country to another, it is frequently a wise procedure to refrigerate the material with dry ice or by other suitable means. This is particularly the case with bacterial and virus diseases in which the diseased insect disintegrates rapidly. Refrigeration not only reduces the amount of autolytic cellular destruction in the insect but also decreases the growth of saprophytic bacteria and fungi, normally present in the gut of the insect, thus preventing these adventitious forms from overgrowing the true infecting agent. The material should never be placed in alcohol or other preservative or fixative unless the specimens are intended only for sectioning. If the insects are alive when sent, it is advisable to maintain the humidity by placing in the container some of their regular food material or a few fresh leaves. Sometimes a little moist soil is helpful, particularly for soil insects. Such precautions may aid in prolonging the life of the diseased insects and in preventing the desiccation of dead insects.

It is usually preferable to ship the insects when they are in the earlier stages of the disease. Small pieces of the food or host plant should be enclosed in such shipments. It is always practical to send entirely normal and healthy insects along to enable the pathologist to make comparisons with the infected specimens. In fact, the ideal shipment consists of (1) healthy insects, (2) insects in the early stages of the infection, and (3) insects moribund or dead of the disease. Each of these groups should be kept well separated from the others.

Certain information should always accompany each shipment. This includes (1) the scientific and common names of the insect when known, (2) the exact locality at which it was collected, (3) the extent of the disease outbreak and the conditions under which it occurred, (4) the name of the collector, (5) the date collected, and (6) the name of the host plant or animal. Other pertinent information may be enclosed, but these six items

are the essential minimum. When this information arrives at the laboratory, along with the insect specimens, it is recorded in a suitable fashion, preferably in an accession file or book of some kind.

Laboratory Procedures. In the laboratory, the insect pathologist first observes the external appearance of the diseased insects and records a description of any noticeable symptoms. From this point on the procedure followed will depend somewhat on the type of infecting agent suspected of being present. Two general methods of examining the insects may be used.

The diseased insects may simply be crushed or triturated in a small amount of sterile saline with a sterile mortar and pestle. The resulting suspension may then be examined microscopically and cultured. If the material is to be cultured, it is usually a wise procedure, when possible, to sterilize the exterior surface of the insect before comminuting it. Effective sterilization may be accomplished by the use of such germicides as Merthiolate, Metaphen, hexylresorcinol, or a solution of 1 : 1,000 mercuric chloride in 70 per cent alcohol for a few minutes. Care must be taken to remove thoroughly all traces of the chemicals before the specimen is triturated; otherwise they may prevent certain of the internal microorganisms from growing on the culture medium.

A second method of examination consists of careful dissection of the insect under aseptic conditions, followed by the microscopic and cultural tests of the various parts. Examination of the blood can be made by puncturing the body wall or by snipping off an appendage of the insect and catching on a glass slide the droplet of blood which oozes out.

Frequently it is necessary or desirable to make histological sections not only for purposes of diagnosis but to gain a better idea as to the relationship between the microorganism and the insect host. It should be remembered, however, that when tissues are fixed, sectioned, stained, and mounted, profound changes usually occur; proteins are precipitated and spatial arrangements are altered by such things as the shrinking of cell membranes. Nevertheless histological methods usually give us significant information concerning the histopathology of the disease.

The histological methods used vary greatly, but essentially they are somewhat as follows: The insect or tissues from the insect are placed in a solution, called a "fixative," which prevents decay and renders the contents of the cells insoluble. Usually the fixative, after acting, has to be washed out, after which the tissue is impregnated throughout with melted paraffin or other embedding material. This must be done by first extracting the water in the tissue with alcohol or dioxane. If alcohol is used, it is extracted with such substances as xylol, cedarwood oil, or benzene, which mix with both the paraffin and the alcohol. The tissue is then held in changes of

paraffin until the clearing agent has been replaced. The melted paraffin is then cooled into a solid block. This block may then be placed in a microtome, which cuts the block and the embedded specimen into very thin slices or sections. These sections are attached to a glass slide, and the paraffin is dissolved away with xylene, which in turn is washed out with absolute alcohol. This is replaced by weaker solutions of alcohol, and finally the section is immersed in a solution of a stain. After staining, the section is passed through alcohol to xylene once more. A drop of Canada balsam or Clarite is placed on the slide and a cover slip is then dropped upon it and lowered onto the section. This preparation dries and is held firmly in position, making a "permanent" mount ready for microscopic examination.

Examination of Microorganisms. One of the most important phases of the laboratory procedures in insect pathology is the examination of the microorganisms that may be responsible for the diseased condition. The details of the microbiological techniques employed in examining the diseased insects may vary according to whether one is concerned with viruses, bacteria, fungi, or protozoa. All these forms of life, except the viruses, may be seen with the aid of an ordinary compound microscope.

In the case of the viruses, many of those found infecting insects are accompanied by characteristic inclusion bodies, known as "polyhedra," which first appear in the nuclei of the diseased cells. These polyhedral bodies are readily observed in smears or wet mounts of the diseased tissue. They are best observed in relation to the diseased tissues and cells in histological sections. None of the viruses has been cultivated on artificial media.

Bacteria are best studied in pure cultures, although they may be observed directly in the tissues of the diseased insect by means of histological preparations or in stained smears. Not all bacteria are readily cultivable on artificial media, but most of them are. There is a wide variation, however, in the kinds of media employed. Most of the common forms of bacteria grow well on ordinary nutrient agar, but frequently enriched media must be used. After the causative bacterium is isolated on an artificial medium, its identity is usually determined by noting its reaction in various differential media and solutions, and occasionally by serological comparisons.

Entomogenous fungi, including yeasts, are frequently difficult to grow on artificial media. Many have been cultivated, however, and these can be identified and studied by the usual techniques used by mycologists for such purposes. Identification of the noncultivable forms is usually slightly more difficult. In both cases, identification may rest on a purely morphological basis, and usually it is necessary to see more than one stage to obtain a complete knowledge of the true systematic location of the fungus.

Protozoa may nearly always be recognized and identified by an examination of their morphological characteristics. Some of them have been cultivated on artificial media, but most of them have not. Wet mounts as well as stained preparations should always be made. Since many species of protozoa have rather complicated life cycles, it is frequently necessary to examine protozoa-infected insects at intervals in order to catch all stages of the parasite.

It is well to keep in mind that the condition of the ailing insect and the accompanying pathology need not necessarily be brought about by an infectious condition. Indeed, microorganisms may not be involved in the malady at all. In most of these noninfectious conditions one must sooner or later resort to the use of histological sections to determine the true nature of the condition and its pathology.

Disease. As with the phenomenon of disease in human beings, so it was with the diseases of insects: early observers ascribed the afflictions they saw to a variety of causes. Frequently these causes were considered to be of a miasmal or affluvial nature; or they were attributed to changes in atmosphere, weather conditions, invisible emanations, exhalations, and the like.[1] It is known now that some of these supposed causes actually may be predisposing factors in certain diseases, but it was not until the proper intellectual climate fostered the germ theory of disease that microorganisms were considered as possible agents of the diseases of insects.

Our concept of disease as it applies to insects is essentially the same as that which applies to other animals, including man. In most cases, the word "disease" literally means "lack of ease" and signifies a departure from the state of health or normality. A healthy insect is one so well adjusted to its environment that it is capable of carrying on all the functions necessary for its maintenance, growth, and multiplication with the least expenditure of energy. A diseased insect is simply one that is not healthy or normal. It is an insect that can no longer tolerate an injury or hardship without having an abnormal strain placed upon it. Disease is a condition or process that represents the response of an insect's body to injury or insult. The entire body of the insect need not be in a pathic state. Only a single cell or a single tissue or a single organ may be involved, and this may or may not affect the survival of the insect host. Just how these effects or changes may come about will be discussed more fully in the chapter on infection.

Two large and general categories of disease are usually recognized:

[1] For the reader who may be interested in pre-germ-theory concepts of diseases affecting insects, reference to this subject is made by Aristotle in his "Historia animalium," and the account presented by Kirby and Spence (1826) will be found to be of exceptional interest.

infectious diseases and noninfectious diseases. An *infectious disease* is a disease resulting from the presence of a living microorganism. Examples are pneumonia, tuberculosis, influenza, soft rot of crucifers, foulbrood of bees, and milky disease of the Japanese beetle. The infectious diseases will constitute the greater part of the subject matter of this book.

A *noninfectious disease* may be thought of as any ailment in which a living microorganism is not involved. It may be due to any one of a variety of agencies, including mechanical, physical, chemical, and biological factors. Examples are trauma, rickets, vitamin deficiencies, certain types of cancer, and the like. In a general sense, the noninfectious diseases of insects may be considered as injuries and as noninfectious conditions other than injuries. They may be grouped as follows:

1. Injuries
   a. Mechanical injuries (*e.g.*, traumata, bruises, torn tissue, etc.)
   b. Injuries due to physical agents (*e.g.*, burning, freezing, drought, etc.)
   c. Injuries due to poisons or chemical agents (*e.g.*, insecticides)
   d. Injuries due to parasitization or infestation by other insects or arachnids
2. Noninfectious conditions other than direct injuries
   a. Diseases due to a deficiency of proper nutriment, vitamins, etc.
   b. Diseases due to deranged physiology and metabolism

In other words, disease and its accompanying pathologies may assume a multitude of forms and degrees. It might be helpful, at this point, to consider the number and variety of diseased conditions that can affect a single insect species. The list, as compiled by Fyg (1939), of abnormal conditions to which the queen honeybee is subject may be used as an example (a few additions have been made):

Malformation and uneven chitinization of bees in queen pupae
Wing atrophy
Atrophy of the tarsal segments
Abnormal wing venation
Microcephaly
Defective abdominal chitin
Rudimentary ovary
Asymmetrical formation of the ovary
Duplication of both ovaries
Accessory ovarian follicle in the body cavity
Persistence of the ovarial cord
Rudimentary egg tube
Hereditary lack of the seminal vesicle
Duplication of the seminal vesicle
Dichotomy of the alkaline gland
Hermaphroditic queen
Arnhart's black-egg disease
Parasitic melanosis of the sex organs
Bacterial melanosis of the sex organs

Atrophy of the ovaries
Gontarski's parasitic atrophy of the ovaries
Diseased drone brood (ringed sperm)
Degenerate sperm
Disease of the seminal vesical wall
Obstruction of the sex duct through mucous and sperm masses
Obstruction of the sex duct through egg packets and disintegrated egg masses
Nosema disease
Excrement congestion in the rectum
Actinic mycosis of the rectum
Bacterial ulcer in the rectal epithelium
Parasitic melanosis (H-type) of the rectal epithelium
Wartlike formation in the rectal epithelium
Tumor of rectal papillae
Solidification in fecal vessel (intestinal solidification)
Excrement stoppage
Solidification (nephridial) in the Malpighian tubes
Abnormal excretion in the Malpighian-tube epithelium
Abdominal edema
Hypertrophy of the fat bodies
Parasitic melanosis (H-type) of poison vesicle and poison tube
Hypertrophy of the alkaline gland
Paralysis phenomena after bee sting
Mermis albicans Siebold as a coelom parasite
Acarine disease (*Acarapis woodi* (Rennie))
External mites, *Acarapis externus* M. and *A. dorsalis* M.
Bee lice (*Braula coeca* N.)
Diseases of the brood (American and European foulbrood, etc.)
General mycoses
Two queen nymphs in one queen cell
Phenotypic transitory formation between queen and worker
Dwarf queen
Inverse position of poison vesicle and alkaline gland
Inverse position of the gut
Persistence of the ovarian pelvic wall
Penis obstruction in the genital orifice
Laying derangement as a result of perforation of the anal papillae by queen's own stinger
Thoracic pigment defect
Catalepsy
Egg sterility
Addled brood
Queens that are incapable of producing drone brood
Albinism of the drones (hereditary condition)
Cyclops (hereditary condition?)
Hermaphroditic formation of worker bees (hereditary condition?)

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CHAPTER 2

MECHANICAL, PHYSICAL, AND CHEMICAL INJURIES

The types of injury to which insects may be subjected are many and varied. To be sure, any type of abnormal or destructive alteration of healthy tissue may be considered an injury even when it is caused by an infectious microorganism. The term as used in this chapter, however, has the meaning most often given it, *i.e.*, damage or harm due to specific agents or agencies other than microorganisms. The various kinds of injury may be separated into the following groups: (1) mechanical injuries, (2) injuries due to physical agents, (3) injuries due to chemical agents or poisons, and (4) injuries due to parasitization or infestation by other insects or arachnids. These categories are purely arbitrary and are used for the sake of convenience only.

1. MECHANICAL INJURIES

The mechanical injuries that may affect insects are usually of two types: (1) distention and (2) trauma.

Distention. This injury may result when some duct or hollow viscera is obstructed, thus preventing the outflow of its contents, such as an obstruction of the alimentary tract or of the Malpighian tubes. Although it may occur frequently, this condition is rarely seen in insects, undoubtedly because of the few examinations made for it.

Trauma. Wounds or injuries due directly to violent contact of external objects with the body of the insect are called "traumatic." There is a great variation in the gross appearance of these lesions, but they have similar microscopic structure. The effect is generally one of cutting, crushing, and tearing of the tissue elements, frequently accompanied by the oozing of hemolymph. Wounds usually affect the insect by (1) damaging important organs or tissues, (2) causing hemorrhage, (3) enabling infection to gain a foothold. From the standpoint of heredity or adaptive variation, these injuries are of only minor importance since they have very little or no effect upon the germ cells.

Trauma may be of various kinds. *Bruises* are disrupted or discolored areas produced by injurious contact with blunt objects. It should be remembered, however, that because of the exoskeletons of insects this type of injury does not have the manifestations we usually think of in the

A B C

Fig. 3. Cross sections of tumorous growths in *Leucophaea maderae* Fabr. produced by the interruption of the recurrent nerve supplying the organ involved. *A*. Tumor in wall of salivary reservoir. The border between the thin wall of the salivary reservoir and the tumor is indicated by the arrow. Three layers may be distinguished. An outer layer, consisting of small cells; a middle layer, of swollen vesiculated cells; an inner layer, facing the lumen of the reservoir and consisting of brown cellular debris. *B*. Tumor in salivary gland. Normal glandular tissue and salivary ducts (at right) above arrow. Tumor mass with remnants of glandular tissue beneath arrow. The tumor cells show whorl formation with degeneration in center. *C*. Tumor in wall of foregut. Above and beneath tumor the wall is normal, consisting of a chitin-covered epithelium facing the lumen (to the left) and a muscularis. The stratification of the tumor is pronounced. (*From Scharrer, 1945.*)

case of animals with endoskeletons. *Concussions* are injuries caused by jarring and usually result in functional disturbances. The application of pressure to part or all of the insect body, disrupting internal and connective tissues, is known as *crushing*. It may be of sufficient magnitude to rupture the integument also. *Cutting* refers to wounds (cuts) produced by sharp instruments and usually shows smooth surfaces without much bruising. Vital organs or tissues may be severed; and, if sufficiently severe, too much hemolymph may be lost (hemorrhage), resulting in the death of the insect. Amputations of important structures, such as antennae, may to a considerable extent alter the insect's behavior. *Tearing* indicates the violent pulling apart of tissues, resulting in torn or lacerated wounds. Dislocation of leg joints and bites of other insects may be of this type. *Punctured* wounds are produced by pointed objects, such as needles and thorns. These wounds are relatively deep and narrow.

Traumatic injuries and the mortalities caused thereby may be of particular importance in insectaries where large numbers of insects must be handled rapidly and sometimes roughly. Similarly, the rearing of beneficial insects such as silkworms has been known to be handicapped by injuries resulting from excessively rough handling of the insects. In studying sectioned tissues of insects one must keep in mind the histopathologies that may be the result of these kinds of injury.

The pathologies of experimental injuries have been studied in only a few instances, but some of these are exceedingly interesting. The work of Scharrer (1945) on experimental tumors produced by nerve sections in insects (*Leucophaea maderae* Fabr.) is a case at point. Although tumors may occur naturally as the result of disturbances in cellular metabolism, this investigator found that the interruption of the recurrent nerve in the vicinity of the corpora cardiaca and corpora allata caused tumorous growths in organs that the nerve supplies (anterior portion of the alimentary canal and the salivary reservoir). She was able to produce these tumors in adult males and females as well as in nymphs. The tumors appeared as well-defined conspicuous tissue masses that, in advanced stages, may be seen with the naked eye. Histologically, the tumor consists of layers of cells, the appearance of which becomes progressively abnormal as the tumor enlarges. Distinguishing characteristics serving to differentiate the consecutive layers are commonly present. Usually there are differences in the form of nuclear, as well as cytoplasmic, degeneration. Insects with such tumors die at intervals of from 10 days to several months following the nerve section. Since the anterior portion of the alimentary canal is frequently filled with an abnormally large amount of food at the same time that the fat body is depleted, Scharrer suggests that death may be due to starvation.

Concerning the general occurrence of tumors in insects there is only fragmentary information. Kirby and Spence referred to these malformations in their early work of 1826 and included certain external "blisters" in the same category. Brain tumors have been reported in ants, and hereditary tumors in *Drosophila*. Tumors stimulated by the activity of a polyhedrosis virus occur in larvae of the European spruce sawfly. Undoubtedly other examples exist, but searching for the natural presence of tumors in insects would be like looking for the proverbial

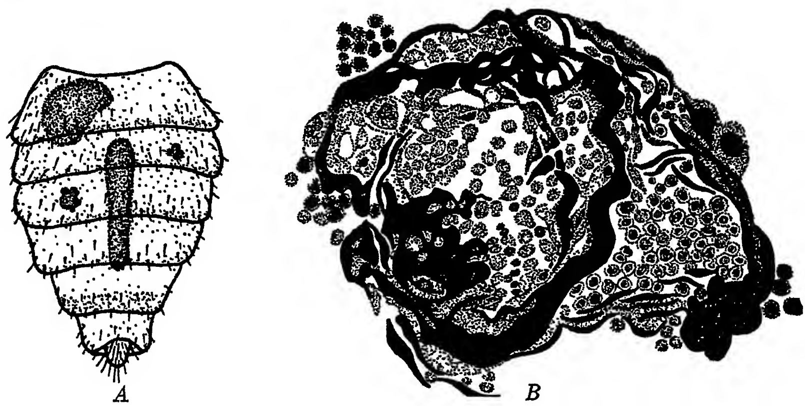

Fig. 4. Tumors hereditary in drosophila flies. *A*. Dorsal view of abdomen, showing tumors. Smaller tumors are regarded as metastases. These may be carried into the heart with the blood, where they develop into a narrow elongated tumor as shown. *B*. A section through the center of a tumor in a late stage of development. (*Redrawn from Stark, 1919.*)

needle in the haystack. Then too the average investigator probably would not recognize a tumor as such if he did see one.

Experimental injury to certain sense organs is known to result in correlated changes in the associated nerve tissue. For example, the reduction or removal of ommatidia from the eyes of drosophila flies results in the hypoplasia (defective or incomplete development) of the optic glomeruli and the elimination from them of certain of the histological traits (Power, 1943).

Healing of Wounds. Wounds are not necessarily fatal to insects; in fact, in the majority of instances, regardless of their age they are capable of repairing such injuries to their tissues. In most insects the reaction to the injury is of two kinds: the accumulation of hemocytes and the reaction of the epidermal cells. In some insects the accumulation of hemocytes is accompanied by a clotting of the hemolymph. Many of the essential facts concerning these processes have been established by Wigglesworth through his studies on *Rhodnius*.

Within a few hours after an incision is made or a wound inflicted, the hemocytes collect along the cut margin. In the course of a day or two they may form a solid plug over the perforation, after which they accumulate more sparsely and spread out on the basement membrane. They apply themselves also to the lower surface of the epidermal cells

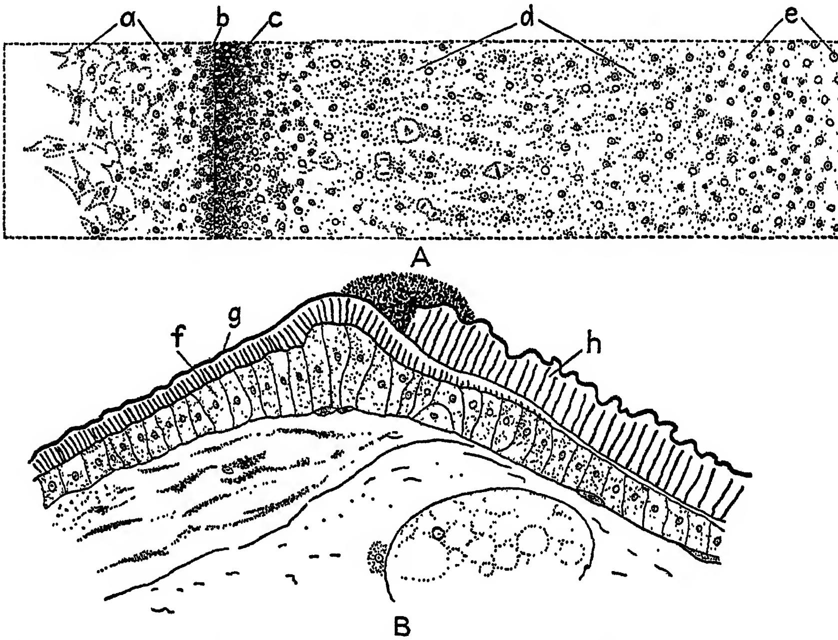

Fig. 5. Wound healing in *Rhodnius* adult. *A*. Surface view of the hypodermis 4 days after a piece of cuticle had been excised. *a*, hypodermal cells spreading over the gap; *b*, margin of excision; *c*, cells heaped up along margin of wound; *d*, sparse zone depleted of cells by migration to the wound, cell divisions occurring; *e*, unchanged hypodermal cells. *B*. Longitudinal section through the margin of a wound after 3 weeks. *f*, hypodermis established under excised region; *g*, new cuticle extending outward under old cuticle; *h*, old cuticle at margin of wound. (*Redrawn from Wigglesworth, 1937.*)

as these spread over an excision. Certain of the hemocytes may also function as phagocytes, disposing of debris and tissue fragments. In incisions not great enough to penetrate the basement membrane, the hemocytes may not accumulate; but, as happens in the case of deeper wounds, the epidermal cells do react. In fact, the main processes in the healing of nearly all external wounds are assumed by the epidermal cells. According to Wigglesworth, the dead or injured epidermal cells in the course of their breakdown apparently give rise to substances, products perhaps of the hydrolysis of proteins, which exert an attraction upon the surrounding cells so that these migrate to the wound and congregate

thickly around its margin, leaving a peripheral zone where the epidermal cells are very sparse. Wigglesworth found that when a piece of the integument has been removed, these aggregated cells spread across the wound, make good the defect, and lay down a new cuticle composed of the usual cuticulin and chitin. In the meantime, cell divisions take place in the sparse peripheral zone, and these continue until the normal density of the cells has been restored.

The phenomenon of regeneration is frequently seen in insects. In growing insects, when appendages are removed, they will reform at later molts. This regeneration usually takes place only at the time of molting; sometimes the injury itself provides a stimulus for renewed molting. Occasionally regeneration does not take place in the larva but is delayed until the pupal stage.

Not to be overlooked is the fact that some insects, *e.g.*, ants, tend their wounds with their mouthparts. This probably has more of a palliative than curative effect.

2. INJURIES DUE TO PHYSICAL AGENTS

Many agencies might be considered to be of a physical nature, and many physical agencies are known to affect insect life. As far as those which sometimes cause pathological states in insects are concerned, however, only a few of the more important, such as heat, cold, moisture, and desiccation, merit consideration at this point.

Heat Injury. In nature, insects are generally able to escape heat injury by virtue of their sensitivity to and consequent avoidance of high temperatures. In most insects, the antennae appear to be the parts of the body most sensitive to heat stimuli. Certain insects, such as grasshoppers, are at least slightly sensitive to heat over almost the entire body surface; but, as noted by Geist (1928), the proximal half of the antennae, as well as the pulvilli and tarsi of the hind and forelegs were the parts most sensitive.

The thermal death points for most insects usually range between 39 and 54°C., although some insects, such as those infesting dried stored products and certain desert insects, are able to withstand temperatures in the neighborhood of 60°C. The maximum temperature tolerated by insects probably is not much over 63°C. Of course the amount of moisture present may make a considerable difference in the degree to which an insect can tolerate a high temperature. Ordinarily insects have a greater resistance to high temperatures when in a dry atmosphere unless this situation is so prolonged that the insect's tissues become desiccated. The time of exposure is another important factor.

Excessive heat may cause the following visible effects: (1) localized

burns causing death of protoplasm in that area (when this injury is caused by moist heat, *e.g.*, boiling water, it is called a "scald"); (2) discomfort and increased irritability and movement; (3) paralysis and cramping of the legs; (4) heat stupor or heat rigor; (5) death. These are usually accompanied by more or less distinctive histological changes in the various tissues involved. For example, nerve tissue may fail to stain characteristically with toluidine blue and will appear coagulated.

Before death from heat takes place, the insects become motionless and may draw their legs up tightly against their bodies, or, if larvae, they become fully extended but still react by twitching when pressed between the fingers or otherwise stimulated. Sometimes the reaction up to this point is reversible and sometimes it is not. For example, Hopf (1940) found that normal flies will seldom develop from *Calliphora* larvae after heat rigor has set in. On the other hand, recovery is general in those of *Phormia* if the rigor has not lasted too long. Under certain conditions the larvae of some insects may to all appearances recover from the heat injury but die within a few days. In such larvae, Jefferson (1945) observed the uptake of basal oxygen to be much greater than in untreated controls, and this was accompanied by a browning of the tissues about a day after exposure to the heat.

The cause of death from heat injury is not known with certainty. At one time it was supposed that death was due simply to the coagulation of proteins in the cell protoplasm. More recent theories have included such reasons as the destruction of enzymes, asphyxiation, or some other disturbance in the equilibrium of the protoplasm through the accumulation of waste products. The lipoid-liberation theory appears to be receiving considerable support in recent years. This theory, the first proponents of which were Heilbrunn and Belehradek, attributes death from heat injury to a liberation of protoplasmic fats from the tissues of the animal. Fraenkel and Hopf (1940), however, believe that heat resistance cannot depend solely upon such a phenomenon, and Hopf (1940) showed that exposure of certain flesh-fly larvae to high temperatures produces an increase in certain phosphatides that may be connected with buffering and coenzymatic activities. This increase in phosphatides is connected with a series of metabolic processes instigated by the reaction of the organism to high temperatures and therefore, up to a certain point, constitutes an adaptation of the organism to the change in environmental conditions. When the heat injury is severe or when rigor sets in, it appears that enzyme activation occurs in the hemolymph and there is a general upsetting of enzyme balance in the tissues.

A suggested link between the "enzyme" and the "lipoid-liberation" theories has been provided by Jefferson (1945), who postulates that heat

injury may be due primarily to a breaking up of the mitochondria in certain tissues. He finds that the mitochondria in the fat body of *Calliphora* larvae injured by heat are small discrete globules, while those of the untreated controls are generally larger and often aggregated into clumps. Since mitochondria are thought by some observers to be bound up intimately with enzyme activity and since lipoidal complexes are an important part of the chemical composition of mitochondria, Jefferson suggests that the "liberation" of mitochondria lipoids might result in an upsetting of the enzyme systems of an animal and lead to irreversible heat injury.

Cold Injury. When an insect is exposed to a rapidly falling temperature, its own internal temperature falls to a point where ice crystals are formed in the tissue fluids. This point is spoken of as the "undercooling point" and the temperature as the "undercooling temperature." Just at the time the undercooling point is reached the first ice crystals are formed and the heat of crystallization causes a sudden rise in the body temperature of the insect; the temperature thus reached is called the "rebound temperature" or the freezing point. Actually the true freezing temperature is somewhat higher than the rebound temperature, since some of the heat of crystallization is transferred to the immediate surroundings. This temperature is held constant for a moment or two and then, after the body fluids solidify, falls again to the temperature of the surrounding environment. Thus the temperature of an insect may, for example, fall to − 16°C., after which it suddenly jumps up perhaps to − 2°C., after which it proceeds to fall once more. Although some workers have reported the survival of certain insects after two or more successive undercoolings, most recent work indicates that one undercooling is fatal for most insects. Mechanical injury may diminish the insect's capacity for undercooling. Piercing an insect may raise its undercooling temperature from − 20°C. to − 10°C.

Just how freezing kills an insect is not definitely known, but several theories on this point have been advanced. In the case of those insects accustomed to warm surroundings, such as certain tropical insects that die even at temperatures considerably above the freezing point, death may be due to the accumulation of toxic products ordinarily eliminated at normal temperatures or perhaps to the inability of the insect at low temperatures to utilize certain of its food materials. Most insects die when their tissues freeze, and the cause of death may be the dehydration of tissues or the mechanical injury of the cells by the formation of ice crystals, although the latter theory has now largely been abandoned. Oxidation systems of the cells are also inactivated by freezing, and the normal functionings of the cell walls are believed by some to be destroyed.

Some insects survive complete freezing but die only when the temperature is lowered below their undercooling point. The cause of death in these cases is not understood.

In general, authorities agree that most insects cannot survive temperatures much lower than $-20°C$. Some insects may be "hardened" to low temperatures by gradual subjection to falling temperatures. Such ability to tolerate low temperatures is believed to depend upon the proportion of bound water to free water in the insect's tissues. The bound water is that water which is adsorbed to the hydrophilic colloids of the insect's protoplasm. Gradual subjection to lower temperatures increases the amount of bound water in an insect's tissues, and such water does not freeze at temperatures above $-20°C$.

Although the mortality due to freezing or low temperatures is beneficial in reducing the numbers of certain insect pests, it is something to be avoided as far as beneficial insects, such as bees, are concerned. Occasionally in early spring a colony of bees will expand its brood area beyond its ability to keep the brood warm during a sudden cold spell. If the cluster is forced to contract, the exposed larvae may die of chilling or starvation (Eckert, 1947). Such conditions usually clear up rapidly soon after moderate temperatures return. It has been observed that exposure of queen bees to cold temperatures causes injury to an extent that their colonies soon start to supersede them. This injury appears to be centered largely in the acid gland, which often turns black in various portions (Eckert, 1940). The injury may not be evident until some weeks after exposure, although occasionally the color change is noted within a few hours. At the same time, the poison in the poison sac may become hardened and discolored. The exact nature of the physiological disturbances involved is not known.

Humidity and Moisture. For the proper functioning of their life processes, insects require certain amounts of moisture in their environment just as they need certain degrees of warmth. Actually the two factors moisture and temperature are so closely correlated with respect to the development and activities of the insect that it is difficult to consider them separately. Nevertheless moisture alone may play an important role in the life of an insect.

Some insects are quite sensitive to changes in the moisture content of their environment and have become adapted to rather narrow optimum ranges. Many such insects choose their resting places according to the humidity. Others, such as certain sap-feeding insects, appear to be fairly independent of the influence of atmospheric moisture. In certain cases the rate of the insect's development (*e.g.*, pupae of *Lucilia*) is retarded

at high humidities. It is generally supposed that insects which have an adequate supply of moisture available in their food are relatively independent of the moisture content of their surrounding environment.

From the standpoint of pathology, excess humidity may cause two general types of disorder: (1) waterlogging of tissues, and (2) drowning or suffocation due to deprivation of the air source. Abundant rainfall may provide such adverse conditions for some insects. The larger larvae of certain insects become stupefied within a few minutes or hours after being immersed in water. Pupae may also be unable to withstand prolonged immersion, but some adults are able to survive for longer periods of time. As the expression "weak as a rained-on bee" would indicate, excessive moisture or rainfall is also a hindrance to the activities of most terrestrial insects. However, most of the ill effects arising from changes in moisture content occur by reason of scarcity or absence rather than of abundance.

Drought or Desiccation. The amount of water in insects generally varies from 50 to 90 per cent of the total body weight. The ability to withstand a reduction in this water content may vary with the insect. The reduction usually takes place by evaporation mostly through the tracheal system but also through the body wall when the permeability of this structure is increased by high temperatures or by injury to the epicuticle. When the spiracles are forced, by high temperatures or by the increased activity of the insect, to open more frequently than normal, the amount of evaporation is greatly increased. An insect is frequently able to protect itself against damaging desiccation by closing its spiracles and by its ability to withdraw into its body proper nearly all the water from the contents of its rectum before the elimination of the excreta.

When insects held in dry air die, desiccation is one of the factors causing death, but it usually is not the only factor. Increased temperatures and starvation may also be instrumental in bringing about the ill effects. The movement of the insect is also important under these conditions, because this affects the rate of evaporation. The survival of some insects appears to be more dependent upon humidity changes than for other insects. Ludwig (1937) noted, for example, that the time of survival of the larvae, prepupae, and pupae of the Japanese beetle varies directly with the humidity, other conditions being constant, whereas in the case of certain grasshoppers the time of survival is the same regardless of the humidity.

Some writers believe that a lessened water content in insects usually depresses metabolism and retards development. In this connection mention might be made of one of the theories of Roubaud explaining

hibernation and estivation in insects. According to this worker, in some insects uremic intoxication (asthenobiosis) results from a progressive inability of the Malpighian tubes to eliminate the uric excretory products which, instead, accumulate in the adipose tissues. This inability may increase from one generation of insect to the next. Finally, after several generations, the intoxication so affects the insect that its further development is inhibited to the extent that hibernation or estivation follows. At this time a prolonged exposure to low temperature or low humidity reduces the metabolism to a lower level, and the accumulation of toxic waste products is eliminated through the Malpighian tubes. After this elimination, the processes of development will be restored.

Desiccation during the time an insect is in the pupal stage is considered to be the cause of certain types of dwarfed or crippled adults. One such injury is the emergence of adults with crippled wings. In nature, as high as 25 per cent of emerging adult butterflies in a given area have been observed to suffer crippled wings during an unusually dry summer or fall.

The effect of desiccation on insects may occasionally be of a purely mechanical nature. This is exemplified by the fact that the chorion of the egg may, through desiccation, become so hard that the insect is unable to break through. Similarly, insects about to emerge as adults may not have enough water in their blood to give it sufficient volume for rupturing the pupal case.

Other Factors. Several other relatively minor physical agencies may cause injury or death to insects. Most of these, however, are correlated with those factors already discussed—temperature, moisture, and desiccation.

It is conceivable that the lack of a proper supply of oxygen may give rise to certain pathological conditions. Oxygen is needed for the normal life of all insects, although some (*e.g.*, cockroaches) are capable of living for long periods in the absence of atmospheric oxygen. The lack of oxygen may inhibit the development of certain insects, and it is probably directly injurious when the oxygen supply fails and the oxygen tension in some particular part of the body becomes zero. Some insects become rapidly anesthetized when exposed to high concentrations of carbon dioxide. Carbon dioxide, even in large amounts, does not necessarily act as a lethal poison. For example, the pure gas does not kill silkworms even after several days of exposure. When, however, the atmosphere contains more than 5 per cent of the gas, the caterpillars lose their appetite, and their growth during early stages is more of less retarded.

Suffocation has been considered to play an important role in the killing of insects by petroleum oils. The more volatile petroleum oils do have

a direct toxic effect, but the nonvolatile oils kill very slowly and, in certain cases at least, largely through the asphyxiation of the insect.[1] Richards (1941) studied the effect of suffocation on insect tissues and found that in the central nervous system the principal histological result is a clumping of the chromatin around the nucleolus, leaving the remainder of the nucleus filled with clear fluid (Fig. 6). This phenomenon may occur in

Fig. 6. Section of a portion of a thoracic ganglion of a cockroach, *Periplaneta americana* (Linn.), showing the histological effects of suffocation (premortem). Note clumping of chromatin in most of the nerve cells, but not in the smaller neuroglia cells (horizontal row at top), the nuclei of which are normal. The effects shown are reversible on return of the insect to aerobic conditions. (*From Richards and Cutkomp, 1945; courtesy of A. Glenn Richards.*)

the cells of other tissues but is more extreme in nervous tissue. Asphyxiation also produces a more reticular chromidial picture, although extensive chromatolysis probably does not occur until post-mortem degeneration sets in.

Light, in itself, may have no disastrous effect on insects except as it is related to other factors, such as temperature and humidity. Certain insects, such as termites, are apparently uncomfortable in the presence of light. The pupae of the bollworm, plum curculio, and other insects are frequently killed upon exposure to hot sunshine. Although it is

[1] At this point it is interesting to recall the statement made by Aristotle in his "Historia animalium" to the effect that "All insects die when plunged in oil, and most rapidly if their head is oiled, and they are placed in the sun."

possible that the ultraviolet rays may exert some deleterious influence, it is probable that the ill effects are due primarily to heat and the accompanying evaporation.

Electric shock and supersonic waves may also injure or kill insects, as is evidenced by the use of these agencies in certain types of insect control. Low-voltage electric current may cause only a temporary local injury. Currents of higher voltage cause their destruction by burning and probably by some destruction of the nervous system directly. Exposure of insects to fields of high radio frequency has been shown to kill them in a very short time and to cause distinctive lesions in the nerves of these animals. Although in some cases it is known that the effect of high radio frequencies on animals is due actually to the generation of internal heat of a lethal degree, it has also been shown that when very short wave lengths are involved, the heat generated may be of little significance. Probably also of significance is the fact that insects killed with high-frequency radio waves histologically show nerve lesions unlike those produced in insects killed by heat applied externally.

That physical agencies may adversely affect the nervous system of insects was of course suspected a long time ago. Considerable ignorance still exists, however, as to the exact nature of much of this adverse effect, except as it has been demonstrated on animals other than insects. It is interesting to know that early writers ascribed certain peculiar behavior of insects to a malfunctioning of the nervous system. Kirby and Spence (1826), for example, ascribed a kind of "vertigo" of ants and other insects to a "derangement of the nervous system."

3. INJURIES DUE TO POISONS

Any substance that injures living cells by chemical means is commonly considered a poison. Poisons may be preformed substances that enter the insect through the body wall, the alimentary tract, or the spiracles and tracheae; or they may be formed in the body itself through the action of bacteria, the disintegration of necrotic tissues, and possibly by the suppressed function of certain tissues and by the perverted metabolism of body cells. Those poisons produced by microorganisms will be discussed in a subsequent chapter.

A complete discussion of the subject of poisons falls in the realm of insect toxicology. A certain part of insect toxicology, however, is concerned with the pathological principles involved in the killing of insects with insecticides. It is obvious that, when an insect is sickened, killed, or otherwise affected by a toxic chemical agent, a pathological state results. This may be readily discernible in the gross appearance of the insect and

its organs, or it may be confined to the derangement or destruction of cellular elements, in which case we are dealing with what is termed "histopathology," or the pathology of tissues and the cells that compose them. The main purpose of including this subject in the present book is to assist in orienting the student with respect to variations that occur between the pathologies brought about by purely chemical agents and those which result from microbial action.

Accordingly, we shall not be concerned here with the nature of the various poisons or with the detailed physiology and chemistry of their modes of action. Instead the discussion will be limited to a brief consideration of some of the symptoms, pathologies, and malfunctionings that may result in the bodies of insects affected by chemical poisons. It should also be kept in mind, however, that sometimes the more subtle effects of these substances escape our observation and that it is often difficult to separate the normal from the abnormal.

Symptoms and Gross Pathologies

Symptoms in Insects Affected by Poisons. Those substances which kill insects directly by their chemical action are usually called "insecticides" by entomologists. They are frequently grouped into three general classes: (1) stomach poisons, (2) contact poisons, and (3) fumigants or respiratory poisons. This grouping is made according to whether the poison enters the insect's body through the lining of the digestive tract, the outer covering or integument of the body, or the lining of the respiratory system. Stomach and contact poisons are generally used on insects infesting animals and when treating plants and products that are situated in the open air. Fumigants are most frequently used when the infested materials are in tight enclosures, rooms, or burrows.

The route of a poison's entrance into an insect does not necessarily dictate the particular part of the animal's body that will ultimately be affected. Neither will the observable symptoms necessarily depend upon the route taken or upon the organ or tissue affected. In general, it is true that the destruction of any specific tissue will finally affect the insect in much the same way regardless of the poison employed. It is not so simple as this, however, since, up to a certain point, each poison or group of poisons usually elicits its own peculiar reaction and produces its own particular pattern of pathological changes; these may be quite complex and may involve several different tissues and systems.

Death of an insect by a poison is usually thought to be due to the disruption of an enzyme system, and some of the symptoms noted before

death occurs may be caused by the earlier processes of this disruption. These symptoms are many and varied, but they usually take the form of an alteration or malfunctioning of some physiological or metabolic process in addition to the direct toxic effects on the cells.

Usually one of the first discernible symptoms is the decreased general activity of the insect but with the retention of its abilities to crawl, walk, fly, and keep its equilibrium. As the effect of the poison increases, these abilities are inhibited, its facility for regaining its equilibrium frequently being the first to leave. When placed on its back, the insect, although unable to right itself, may nevertheless be able to move its legs, other appendages, and body fairly vigorously. Movement of the legs then slows down considerably until only slight movements can be detected. Eventually slight movement can be elicited only by mechanical stimulation. When no response to mechanical stimulation is forthcoming, the insect may be considered dead. This is the arbitrary criterion used by Munson and Yeager (1945) in their studies on the toxic effect of arsenicals on roaches. The actual death of most of the tissues may be somewhat delayed. With such poisons as cyanide nerve tissues die and begin disintegrating first, glandular tissues die shortly thereafter, and it may take several hours for muscle tissues to die. Many tissues are also destroyed by the autolytic and other enzymes which begin to digest surrounding tissue after the gross death of the insect.

Other symptoms may be noted, depending upon the particular poison concerned. For example, there may be twitching, turning, retching, vomiting, or the excretion of feces of abnormal consistency. Mortality may be preceded by a prolonged period during which there is a continuous loss of weight, as occurs in the delayed mortalities of insects (*Pyrausta* and *Sesamia*) exposed to lethal doses of methyl bromide. There may be a change in the color of particular tissues such as those of the alimentary tract, or the hemolymph may become faintly tinged. In some insects, phenothiazine imparts a red color to the hemolymph when this is exposed to the air. Complete or partial discoloration or blackening of the appendages of certain insects occurs when they are poisoned with pyrethrum or with rotenone. There are other similar examples. Typical symptoms of poisoning are seen in insects affected by DDT except that the poisoned insect in many cases assumes a peculiar and almost characteristic tremor not seen in other types of poisoning. According to Bodenstein (1946), the wings and legs of drosophila flies injected with 1 per cent DDT go into spasm long before the muscles of the abdominal wall do. The DDT symptoms can be alleviated or prevented by the administration of phenobarbital, which affects the nervous system, indicating that DDT acts

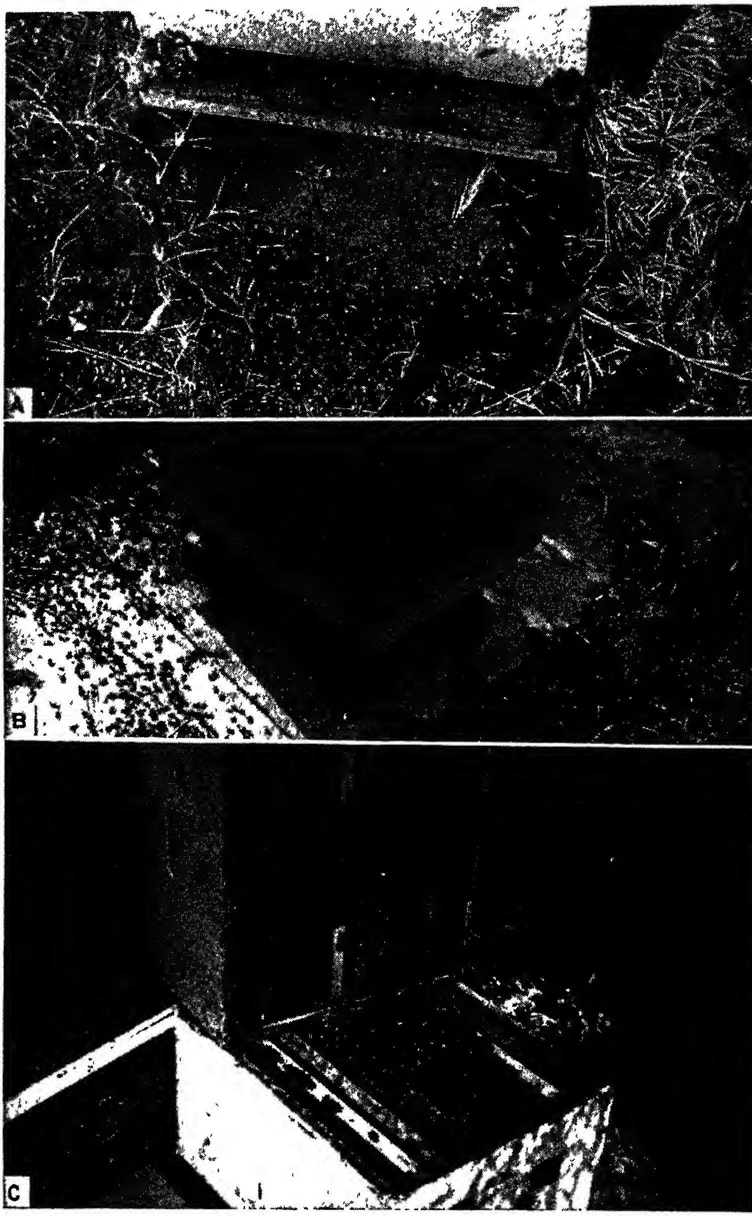

Fig. 7. Views of beehives showing the effects of chemical poisoning as indicated (in *A* and *B*) by the number of dead bees on the ground about the hive. The bees contacted the poison from plants over which calcium arsenate drifted following the dusting of tomato fields by airplane. *C.* A colony of bees greatly weakened by arsenical poisoning. In a healthy colony the bees ordinarily cover all the frames. (*Courtesy of J. E. Eckert.*)

on the nervous system. There is evidence that the primary action of DDT, as well as that of many other insecticides, is physical rather than chemical and that it is essentially a physical interference at the lipid surface of the axon (Welsh and Gordon, 1947).

In the case of colonized insects, poisoning can also be detected by the actions of the colony as a whole. Thus broods of bees poisoned with pollen from fields treated with insecticides and poison dusts usually are affected rather suddenly, and the effects of the poisoning may last over an extended period. The poisoned larvae die in all stages. These characteristics usually differentiate poisoned brood from brood suffering from European foulbrood. The type of poison responsible can frequently be determined by a chemical analysis of the dead larvae, adult bees, and pollen in the combs.

Effects of Poisons on the Circulatory System. Almost any of the several "systems" of an insect's anatomy and physiology may be profoundly affected by the action of a poison. From the standpoint of gross observation, the one that has received the most study in this regard is the circulatory system, particularly the heart. Accumulating evidence indicates that most insecticides visibly affect the circulatory system, although this effect may not necessarily be a direct one.

In most insects the circulatory system consists, essentially, of a *hemocoele* containing the circulating blood which directly bathes all the organs and tissues. There is no complex system of veins and arteries as in higher animals. The hemocoele is usually divided into sinuses by diaphragms or fibromuscular septa. The principal organ of the circulatory system is the *dorsal vessel,* which is usually divided into the heart (the posterior part) and the aorta (the anterior part). This structure is simply a narrow continuous or chambered tubelike affair, the sides of which are perforated with small valvular openings called "ostia." The blood, throughout the body cavity, is kept in motion by the waves of usually forward-moving contractions or pulsations of the heart or, in some cases, of the entire dorsal vessel. When the heart is functioning normally, three phases usually occur: systole (contraction), diastole (relaxation), and diastasis (rest). The rate of heartbeat is dependent upon many factors, including temperature, movement, stage of development, and rate of metabolism. Depending upon the species of insect, it usually varies from an average of 10 to one of 150 beats per minute.

The complete cessation of the heartbeat for any appreciable length of time usually portends the death of the insect. That such inhibition is not necessarily fatal was shown by Campbell (1926), who observed the silkworm to live for several days after its heart was stopped by an injection of eosin solution. Since the blood of most insects is not important in

conveying oxygen to the tissues, the sluggish circulation resulting from the inhibited heartbeat can be fairly well tolerated for a limited time.

Chemical substances affecting the rate and intensity of the contraction of the heart need not be toxic in the general sense of the word. Changes in the salt concentration and pH of liquids in which insects are immersed may cause corresponding changes in the heartbeat. The rates are usually decreased with a reduction in salt concentration and, within limits, are increased by hypertonic solutions. Any appreciable increase in either the acidity or the alkalinity decreases the rate of heartbeat, although the sensitivity to acid is the greater.

The complete cessation of heart action after the application of various insecticides has been observed by numerous workers. Cessation may occur while the heart is in either systole or diastole, depending upon the poison and the species of insect. Whether the stoppage is due to an effect upon the nervous system or upon the heart muscles themselves, or both, has not been clearly worked out. Most of the common insecticides cause the heart to stop beating within a few minutes after they are applied. Using uncontrolled concentrations, Kirschner (1932), for example, found that the nicotine fumes of burning tobacco stopped the heart pulsations of the tulip aphid in 2 to 3 minutes, carbon disulfide in 4 to 7 minutes, ethyl acetate in 5 minutes, carbon tetracholoride in 13 minutes, a mixture of the last two in 11 minutes, and formaldehyde and nitrobenzene in about 1 hour. (See also Davenport, 1949.)

Sometimes the depressing effect of a toxic substance is preceded by a short period of increased rate of heartbeat. The fumigants ethyl acetate, carbon tetrachloride, and benzene may have this effect. So does sodium arsenite when it is injected.

Yeager and his associates have contributed considerably to our knowledge of the effect of poisons on the circulatory system of insects, including the development of numerous ingenious devices, such as the mechanograph, by which such studies could be more readily carried out. Among these studies was that in which it was observed that, as dilute solutions of several organic thiocyanates decreased the rate of beat of the isolated heart of the roach *Blatta orientalis* Linn., there is at the same time a general dilation of that organ. This apparently is due not to a loss of strength in the heart muscles but rather to an increased tonus of the alary muscles which are thought normally to be responsible for the diastole during the cardiac cycle.

That the sensitivity of the hearts of insects to a single poison varies with the species was shown by Yeager and Gahan (1937). These men found that, when isolated heart preparations from the fifth- or sixth-instar southern armyworm (*Prodenia eridania* Cram.) and the American cock-

roach (*Periplaneta americana* Linn.) were perfused with various concentrations of nicotine, the sensitivity of the roach heart was much greater than was that of the armyworm, as indicated by the respective contraction rates. The initial stimulatory and subsequent inhibitory responses of the cardiac mechanism of the roach heart were elicited by a relatively lower nicotine concentration than was required for the same response in the armyworm heart preparations. They also noted that both the stimulatory and the inhibitory effects were essentially reversible.

In some cases, as with arsenite ingested by the larva of the cabbage butterfly, there are indications that the heart is affected before there is any visible damage to the midgut (Hoskins, 1940). After applying derris dust to silkworms, mosquito larvae, diamondback cabbageworms, American cockroaches, or tomatoworms, Tischler (1935) noted that a diminution resulted in the number of heartbeats even though the body movements were still normal but that heart action continued feebly until the death of the insect. Perhaps more frequently, however, cardiac changes occur subsequent to certain other changes in parts of the insect body more directly affected. For example, Krüger (1931) subjected *Corethra plumicornis* Fabr. larvae to aqueous suspensions of pyrethrum powder and noted that even though the insects went into continuous convulsions the heart action was apparently normal for at least an hour after the convulsions had started. Yeager and Gahan (1937) injected armyworms with nicotine and, for certain concentrations at least, observed that the heart action persisted even though other muscles were completely paralyzed.

The volume of blood in insects subjected to poisons also seems to be affected in certain cases. Such substances as arsenic, carbon disulfide, and pyridine apparently reduce the total quantity of blood of cockroaches. According to Hoskins (1940), this condition is likely to arise with any substance that causes hypersecretion by the midgut cells and consequent loss of water in the feces, and also whenever excessive ventilation of the respiratory system occurs. In making a study of the effect of toxic gases on the blood of the cockroach *Blatta orientalis* Linn., Shull, Riley, and Richardson (1932), in addition to noticing the diminution in blood volume, also detected a decrease in the number of blood cells in roaches killed with carbon disulfide but not in those killed with pyridine. Crystals of ammonium phosphate appeared in the blood of roaches that had been killed with ammonia gas. These workers concluded that since these were essentially the only effects observed in the blood of roaches treated with 34 inorganic and organic compounds of widely differing physical properties and chemical composition, it is probable that lethal concentrations of most gaseous compounds do not produce marked visible changes in the blood of the oriental cockroach.

To some extent the blood cells of certain insects subjected to poisons undergo morphological and chemical changes. Since these are essentially microscopic changes, they will be discussed in the paragraphs on the histopathological changes brought about by poisons.

Gross Pathologies of Other Systems. In addition to the circulatory system, other systems or parts of the insect body also are usually affected by most insecticides. In some cases the changes that take place are detectable more through microscopic observations than through any gross changes in appearance. Furthermore, the gross pathologies generally have received so little attention that our knowledge of them is very incomplete. A few examples of the types of gross pathologies that have been reported should, however, be mentioned.

Poisons entering the insect through the respiratory system may do so without visibly affecting the tracheae themselves. On the other hand, certain insecticidal oils are thought to rupture, collapse, or otherwise disrupt the tracheae or tracheoles. Some insecticides, such as derris, may cause complete cessation of respiratory movements and spiracular action.

The digestive system may show very little gross pathology, although there may be a considerable alteration in its physiological functioning. The alimentary tract may show discoloration or may assume the color of the insecticide. The gut may become indented and darkened in the region of the poison's absorption. In some cases the intestine rapidly disintegrates after a poison has been ingested by an insect; occasionally the midgut contracts and the walls are thrown into folds.

As has already been mentioned, the reaction of the nervous system to poisons may be evidenced by relaxations and sudden contractions of the sphincters of the gut, resulting in vomiting or in anal discharges. Furthermore, such reactions as convulsions, spasms, and paralysis are usually the result of direct action upon the insect's nervous system, particularly upon the central nervous system. Nicotine, for example, may bring about an initial exciting stimulation or irritation which is followed by stupefaction and immobility, depending upon the methods of application and the concentrations used. With the ingestion of other poisons, *e.g.*, formalin, an ascending paralysis may set in, with the movements controlled by the cephalic ganglia being the last to disappear. Most of the actual pathological changes noted in the nervous system must of necessity be observed by histopathological methods.

Although the Malpighian tubes are the most important organs of excretion in an insect, other organs and tissues, such as the fat body, may also be involved. Hence the disruption of the function of the Malpighian tubes alone, owing to injury from a poison, is not likely to lead to the rapid death of the insect. As pointed out by Hoskins (1940), however, it is

possible that such failure may so influence the activity, appetite, or fecundity of the affected individual as to be of importance regardless of its ultimate death or recovery. In certain aquatic insects the anal papillae, which may be considered as part of the excretory system, react to toxic agents. Thus Pagast (1936) showed that in a solution of 5 per cent sodium chloride the anal papillae turn gray, their lumina enlarge, and the nuclei disintegrate; in dilute solutions of silver nitrate or potassium permanganate the papillae turn brown and their epidermis is destroyed, but that of the remainder of the body is not so affected.

Most indications as to the occurrence of pathological changes in the reproductive system of insects have been evidenced by their altered physiology or function. Numerous reports have been made to the effect that after exposure to various insecticides certain insects exhibit decreased egg laying, sterile eggs, premature or defective births or hatching, difficulty in metamorphosis, and the like. Sometimes the ill effects appear to carry over to the second generation. Descriptions of gross pathologies of the sexual organs of insects subjected to poisons are extremely few.

Histopathologies

Some of the most interesting observations of the pathological changes occurring in insects subjected to poisons are histopathological in character; *i.e.*, the injury is observable microscopically in the tissues and cells (cytopathology) of the affected animal. Nevertheless, it cannot be said that the histopathology of poisoned insects in general is at all well known or studied. Furthermore, great care must be taken in interpreting histopathological changes to distinguish true pathology from post-mortem changes or changes caused by agencies other than the one being tested. There has been a tendency on the part of many workers to read too much into the changes seen in sectioned material. Once the "true" pathological changes can be distinguished from the "false" changes, histopathological techniques are able to furnish valuable information to the understanding of the effects of poisons on insects.

For the sake of simplification, it may be best to consider the histopathologies of poisoned tissues according to the particular system or organs involved.

Histopathology of the Digestive System in Poisoned Insects. The fore and hind portions of a poisoned insect's digestive tube rarely show any histological changes. This is probably because of the chitinous layer that lines these portions of the gut. Most of the changes occur in the cells of the midgut, which consists essentially of an epithelial lining of cubical or columnar cells, a connective or basement membrane, an inner

circular layer of muscles, and an outer longitudinal layer. Of these, the epithelium is the tissue that usually shows the most profound histological changes.

As would be expected, the changes seen in the midgut tissues of insects vary with the poison used and the particular species of insect concerned. The changes may vary all the way from complete destruction of the epithelium to no perceptible change whatever. These wide variations were noticed by Pilat (1935*a*), who made a study of the histological picture of the midgut of four species of insects after they had fed on leaves poisoned with sodium fluoride, sodium silicofluoride, sodium and calcium arsenites, and Paris green. The insects used in these experiments were larvae of the small tortoise-shell butterfly, *Vanessa urticae* (Linn.), of the gypsy moth, *Porthetria dispar* (Linn.), the cabbage butterfly, *Pieris brassicae* (Linn.), and nymphs of the migratory locust, *Locusta migratoria* Linn.

In the case of larvae of *Vanessa urticae* (Linn.) killed with the first four of the above named poisons, Pilat observed that the anterior part of the midgut, immediately following the esophagus, is entirely deprived of epithelium. The wall of the intestinal tube is represented merely by a connective membrane and the muscle fibers overlying it on the outside. The epithelium is apparently completely destroyed, since no traces of it are to be found in the intestinal cavity. The damage becomes less marked toward the posterior end of the midgut until after a certain distance the cells are found to be intact and apparently normal. In certain specimens the epithelium is intact throughout the entire length of the midgut, but in its anterior part it shows marked vacuolation, which, in number and size, increases posteriorly. Accumulations of a brown substance of varying sizes and forms may be seen in the vacuoles. This probably represents the initial stage of the disintegration of the epithelium.

With *Locusta migratoria* Linn. Pilat was able to show that the destruction of the midgut epithelium is preceded by the exfoliation of the epithelium from the subjacent connective membrane; *i.e.*, the epithelium separates from its basement membrane in large sheets but retains, at least in the beginning, its typical morphological characteristics. After losing connection with its nourishing base and falling free into the intestinal cavity, the epithelium undergoes disintegration and destruction. It should be pointed out that apparently it is only within the period of time during which the action of a poison is in effect that it is significant in the destruction of the epithelium. The longer the poison acts the greater is the area of epithelium destroyed. Extreme destruction of the tissue may be brought about within 7 hours after a dose of 0.08 milligram of arsenic per gram weight is given, but usually the changes occur more slowly. The destruction usually begins at the anterior end of the midgut and gradually

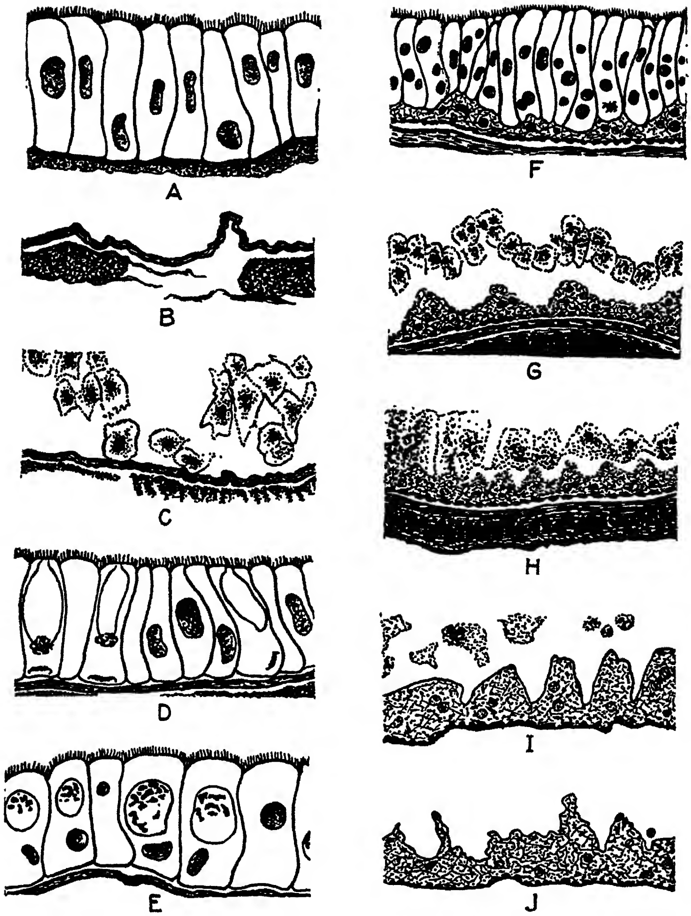

Fig. 8. Diagrammatic longitudinal and cross sections of the intestinal epithelia of insects poisoned by chemicals. *A*. Midgut epithelium of normal caterpillar of tortoise-shell butterfly (*Aglais*). *B*. Midgut of an *Aglais* caterpillar poisoned with calcium arsenite. Basement membrane has been deprived of the epithelium. *C*. Midgut of the same caterpillar farther to the rear, with disintegrated epithelium and exfoliated epithelial cells. *D*. Midgut of same caterpillar, posterior part, with intact epithelium (three cells are of the calciform type). *E*. Midgut of a caterpillar showing symptoms of light poisoning by calcium arsenite. *F*. Diagrammatic cross section of the midgut epithelium of the adult of a normal locust (*Locusta migratoria* Linn.) *G*. Anterior part of locust midgut 5 hours after poisoning with 3 per cent sodium silicofluoride. The disintegrating epithelium has exfoliated in a large linear sheet from the underlying basement membrane. *H*. Posterior part of locust midgut, 12 hours after poisoning with 3 per cent sodium silicofluoride. The epithelium has exfoliated in a continuous sheet from the underlying basement membrane. *I*. Anterior part of locust midgut 7 hours after poisoning with sodium arsenite (dose 0.08 mg.), showing remains of exfoliated epithelium. *J*. Anterior part of locust midgut 96 hours after poisoning with sodium arsenite (dose 0.07 mg.). The epithelium has disappeared, and only the connective membrane is to be seen. (*Redrawn from Pilat, 1935a; very diagrammatic and schematic.*)

spreads posteriorly until the whole midgut is involved. Since the same histological picture frequently results from the use of different poisons, no definite indication as to which insecticide was used can necessarily be obtained. Pilat observed no significant histological changes in the midgut epithelium of larvae of *Porthetria dispar* (Linn.) which had been poisoned with sodium silicofluoride. In larvae of *Pieris brassicae* (Linn.) only slight nuclear changes were apparent.

Results in a general way similar to those of Pilat were obtained by Woke (1940) who worked with larvae of the southern armyworm, *Prodenia eridania* (Cram.). Woke observed that the ingestion of arsenicals was followed by disintegration of the midgut epithelial cells and damage to the midgut muscle fibers. Following the ingestion of sodium fluoride there was disintegration of the substance of the cytoplasm and nuclei of the epithelial cells, and similar marked disintegration followed the ingestion of sodium fluoaluminate. The latter compound also caused some muscle damage, as evidenced by the faint to definite obliteration of the cross striations. No definite histopathological effects were noted after using barium fluosilicate, phenothiazine, or rotenone.

A variety of other histological changes may occur in the digestive tissues of poisoned insects. When a striated border is present, definite signs of its disintegration may be apparent within a few hours after the insect receives even small to moderate doses of poison. The peritrophic membrane may lose its elasticity or be completely destroyed. Evidences of hypersecretion by the epithelial cells may be apparent. The muscle layers surrounding the midgut may show an energetic contraction and separation of its elements, and occasionally actual disintegration.

Of more than passing interest is the observation of Wilson (1936) that the injection of soluble arsenic into the body cavity of *Pieris rapae* (Linn.) in the same amounts as by mouth caused the same destructive changes in the midgut. Since there were no marked histological changes observed in other parts of the body, a special affinity of arsenic for the midgut epithelial cells is indicated.

Histopathology of the Circulatory System in Poisoned Insects. Since microscopic changes in the blood cells of insects may be observed without very much difficulty, the hope has been held that they may serve as an indication of the various types of toxicological reactions brought about by insecticides. Although there is some indication that this may be the case, nevertheless the hopes are still far from being fully realized. The situation is complicated by the fact that so little is known concerning the blood picture of normal insects. As will be brought out in the chapter on immunity in insects, several classifications of blood cells exist and there has not been much success in correlating them. Then, too, there is extreme

difficulty in always being able to differentiate normal from abnormal cells in any particular insect.

Nevertheless a significant beginning has been made by investigators both in this country and abroad. The Russian worker Pilat (1935*b*) studied the effect of poisoning with sodium arsenite and sodium silicofluoride upon the blood cells of the locust *Locusta migratoria* Linn. He found that, with certain exceptions, the influence of the poisons on the blood cells of this insect is far from being definite and that in most cases the blood picture does not present an appreciable deviation from the normal. In some instances, however, disintegration and destruction of the blood cells do take place. Frequently there may be found among the hemocytes very minute cells having a compact and darkly staining nucleus surrounded by an extremely thin layer of cytoplasm. A considerable number of these cells, which may be considered as regenerative forms, may be seen undergoing mitotic division. This process apparently is a reaction of the insect against the poison and is not the result of a direct influence on the formed elements of the blood.

It is of interest to note that Pilat (1935*a*) found the histological picture of the intestinal epithelium of the poisoned insect to correspond with the blood picture in the same insect. If changes appeared in the epithelium of the midgut, disintegration of the blood cells in the hemolymph or the formation of minute cells also occurred.

In 1942 Yeager and Munson reported on the changes induced in the blood cells of larvae of the southern armyworm, *Prodenia eridania* (Cram.), by the administration of poisons. In some of their experiments these workers first fed the larvae on a diet to build up the blood-cell glycogen and, after starving them for 2 hours, ligatured them by tying a string tightly about the body of the larva, separating it into an anterior and a posterior portion. No marked hematological changes were observed to follow the administration of nicotine bentonite, nicotine peat, rotenone, pyrethrum, and phenothiazine. Nor did any of these poisons cause a significant decrease of the mean glycogen index of the fore ends relative to that of the hind ends of the ligatured larvae, although relative decreases in the fore-end glycogen indices did occur following the administration of arsenicals, fluorides, and mercuric chloride. Similarly, marked hematological changes in the fore ends relative to the hind ends of the ligatured larvae followed the administration of the last three named chemicals. These changes, which were progressive, were characterized principally by the agglutination, distortion, and disintegration of the cells and by an apparent loss of the cells from the blood. An increase of mitosis also appeared after the administration of these poisons. The degenerative cytoplasmic changes consisted of apparent cellular swelling, disruption of and decrease

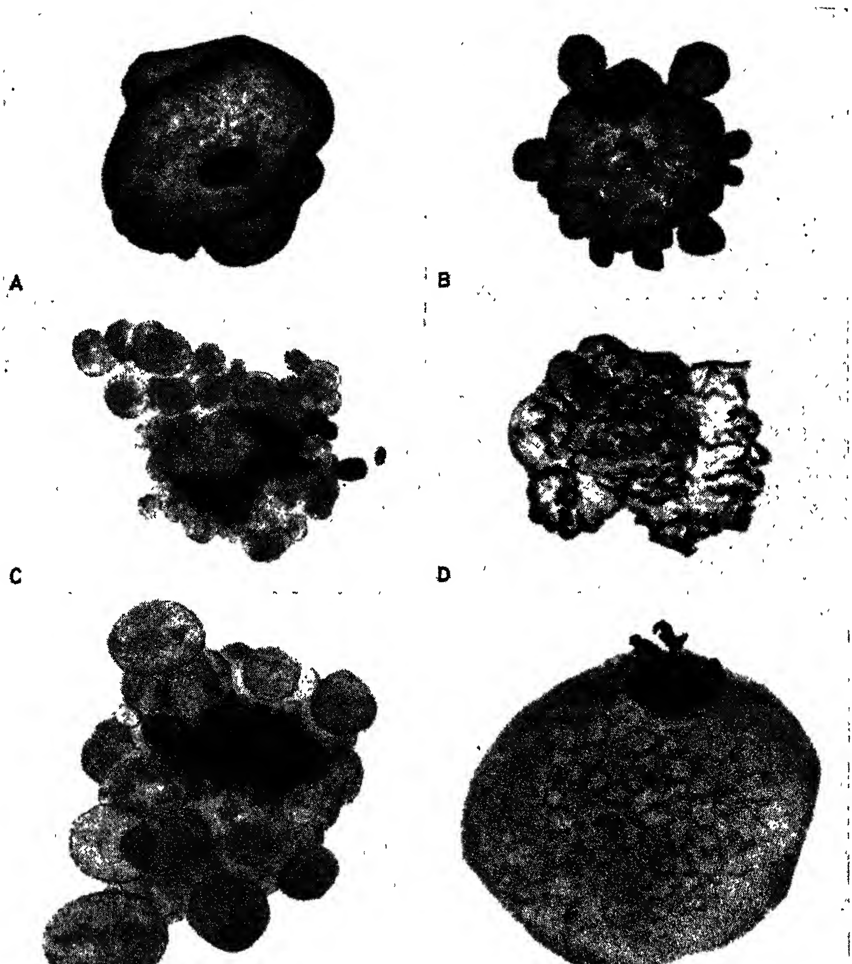

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Fig. 9. Blood cells from poisoned larvae of the southern armyworm, *Prodenia eridania* (Cram.). *A–C*. Blood cells from larvae poisoned with paris green. *D–F*. Blood cells from larvae poisoned with mercuric chloride. *A*. A cystocyte showing the formation of broad pseudopodialike bulges, a somewhat eccentric nucleus with a tendency toward pycnosis. *B*. A rounded cystocyte showing plastid formation, a grossly punctate nucleus, and a remnant of glycogen inclusion in the endoplasm. *C*. A degenerating plasmatocyte, with achromophilic cytoplasm, showing marked plastid formation, a somewhat ragged nucleus, and remnants of glycogen masses. *D*. A degenerating plasmatocyte showing cytoplasmic granulation (formation of minute plastids), cytoplasmic raggedness, and a somewhat distorted and relatively amorphous nucleus. *E*. A large degenerating cell of questionable identification showing marked plastid formation and a nucleus with an aspect suggestive of mitosis. *F*. A large, round, swollen cystocyte showing nuclear extrusion. (*From Yeager and Munson, 1942; redrawn from original drawings kindly loaned by J. Franklin Yeager.*)

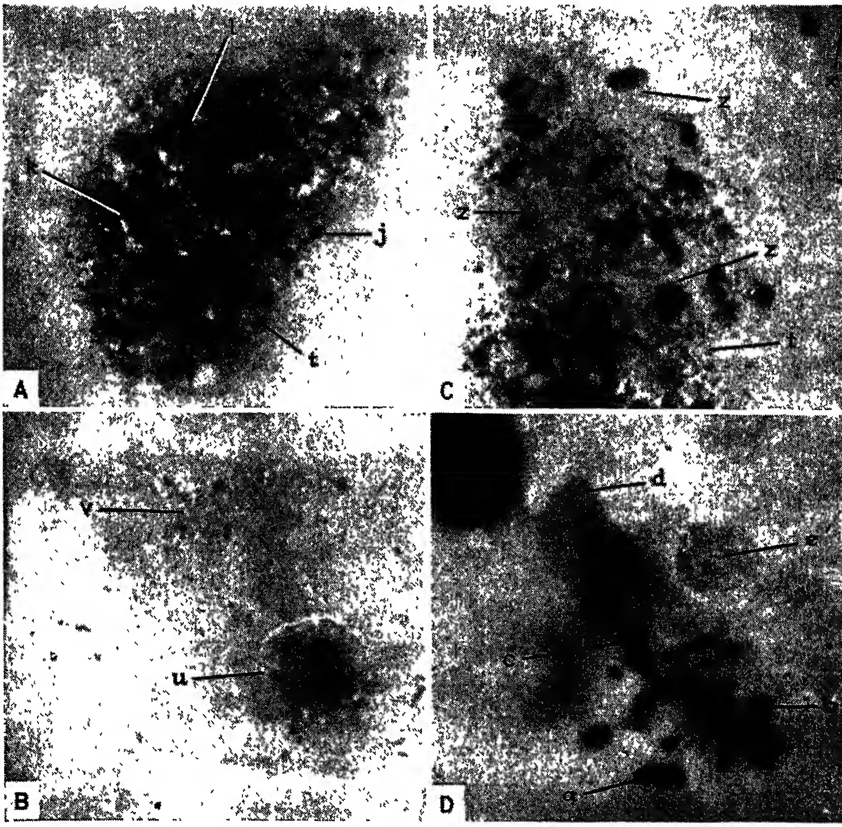

Fig. 10. Changes induced in the blood cells of the southern armyworm, *Prodenia eridania* (Cram.), by the administration of poisons. *A* and *B*. Parts of blood smears from the fore end of ligatured larvae poisoned with barium fluosilicate. *C*. Part of a blood smear from a larva poisoned with calcium arsenite. *D*. Part of a blood smear from a larva poisoned with mercuric chloride, showing nuclear fragmentation (at *a*, *b*, and *c*) and plastid formation (at *d* and *e*). *j*, *k*, *l*, glycogen inclusions; *t*, degenerating agglutinated cells; *u*, degenerating cystocyte; *v*, nearly completely degenerated cell; *w*, degenerating cells; *x*, degenerating cell showing plastid formation; *z*, somewhat swollen translucent cells, probably cystocytes. (*From Yeager and Munson, 1942; courtesy of J. Franklin Yeager.*)

in visibility of normal structure, achromophilia, decrease or loss of blood-cell glycogen, formation of broad pseudopodia or cytoplasmic bulges, plastid formation, excessive vacuolization, and raggedness. Nuclear degeneration involved distortion, raggedness, loss or disruption of normal structure, achromophilia, assumption of a more or less peripheral position, fragmentation, pycnosis, and extrusion.

The significance and fundamental cause of many of these changes in the blood cells of poisoned insects have still to be elucidated.

Histopathology of the Nervous System in Poisoned Insects. The microscopic changes that may occur in the nervous tissue of poisoned insects are extremely difficult of interpretation. Hartzell and his co-workers (1934–1946) have described various distinct changes which they feel characterize the type of destructive action brought about by certain poisons. Richards and Cutkomp (1945), on the other hand, believe that the visible histopathological changes induced in nerves by insecticides are largely post mortem and hence are too complex for analysis with present information, techniques, and methods. Regardless of the variance in views as to the significance of the various histopathological changes seen in poisoned insects, it would be unwise to disregard all the reactions seen as of no value, at least when they are compared with adequate controls. One can make use of such information while at the same time realizing that in looking at killed tissue one is not necessarily seeing the same things that one would be seeing were he looking at living tissue. Furthermore, such histopathological observations may be of considerable significance for diagnostic purposes but may explain very little of the physiology of tissues subjected to the toxic action of poisons. In any case, great care should be exercised in distinguishing between artifacts and true pathological changes known to be caused by the poison. With these points in mind, it would appear worth while to mention very briefly a few of the histopathological changes that have been observed in the nervous tissue of poisoned insects.

Histologically, the central nervous system of insects consists of nerve cells, or neurones, and their fibers surrounded by thin lipoprotein sheaths and held together by tracheae, neuroglia, and a nucleated outer sheath, the neural lamella, or neurilemma. The nerve cell consists of a nucleated cell body and a long filament or axon which usually gives off fine branches and fibrils. Most of the nerve cells and their processes are located in segmental ganglia joined by longitudinal connectives. In stained preparations of nerve cells, characteristic deeply staining bodies known as "Nissl bodies" or as "tigroid bodies" may be seen in the protoplasm. Pathological degeneration of the Nissl bodies is termed "tigrolysis."

Some of the first work on the histopathological effect of insecticides on the nerves of insects was done using pyrethrum as the poison. In 1934 Hartzell described the pathological changes in nervous tissue of adult red-legged grasshoppers, *Melanoplus femur-rubrum* (DeG.), and in larvae of the mealworm, *Tenebrio molitor* Linn., which had been killed by applying pyrethrum concentrates on the dorsal surface of the insects. The nerve tissue was removed and stained with toluidine blue. Cross sections of the

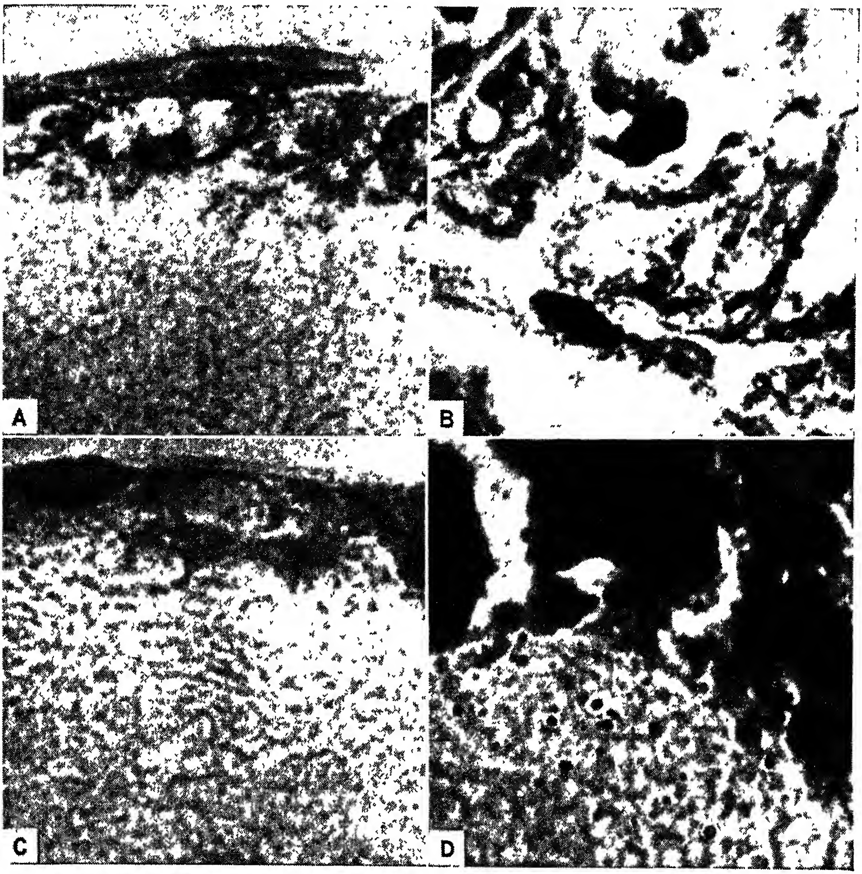

Fig. 11. Effect of pyrethrum concentrate on tissues of the grasshopper *Melanoplus femur-rubrum* DeG. *A* and *B*. Cross sections of the cortical regions of the brains of adult grasshoppers; stained with toluidine blue. *A*. Killed by decapitation. *B*. Killed by pyrethrum concentrate; note the disintegration of the tissue. *C* and *D*. Cross sections of cortical region of thoracic ganglia of adults; stained with toluidine blue. *C*. Killed by decapitation. *D*. Killed by pyrethrum concentrate; note marked disintegration of tissue. (*From Hartzell, 1934; Boyce Thompson Institute.*)

control tissues stained blue throughout. In tissues from poisoned insects, scattered areas among the blue-staining cells stained violet. Other areas were vacuolated and had dark-blue margins. Tigrolysis was also apparent. Such lesions were found in the brain, subesophageal ganglion, thoracic ganglia, abdominal ganglia, and connectives. Triorthocresyl phosphate and γ-thiocyanopropyl phenyl ether produced lesions in the ventral ganglia of the mealworm resembling in most respects those produced by the pyrethrins, whereas rotenone failed to show any appreciable lesions.

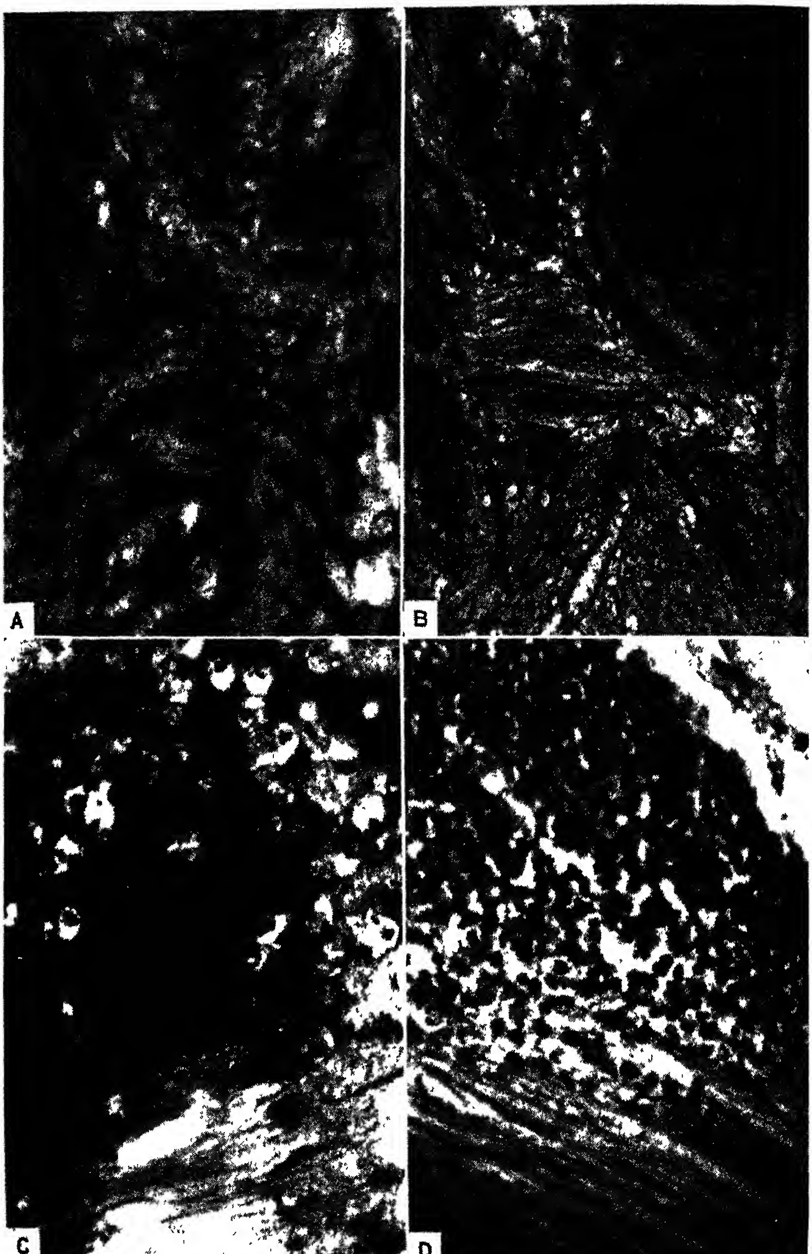
A
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Fig. 12. Histological effects of pyrethrum on nerve and fat tissue of the housefly, *Musca domestica* Linn. *A*. Vertical section through the brain of the housefly, showing the *corpus centrale* (upper right) and the fiber tracts laterad of it. This figure shows the effect of pyrethrum on the fibers. *B*. As in the preceding figure, but untreated. It shows the normal fibers. *C*. Vertical section through the anterior ganglionic mass of the compound eye, showing the effect of pyrethrum on the nuclei. *D*. Normal section of the anterior ganglionic mass. *E*. Normal fat cells from the same region as those of *F*. *F*. Fat body of the head showing the chromatin clumping and clear layer inside the nuclear membrane as a result of pyrethrum treatment. (*From Hartzell and Scudder, 1942; courtesy of A. Hartzell, Boyce Thompson Institute.*)

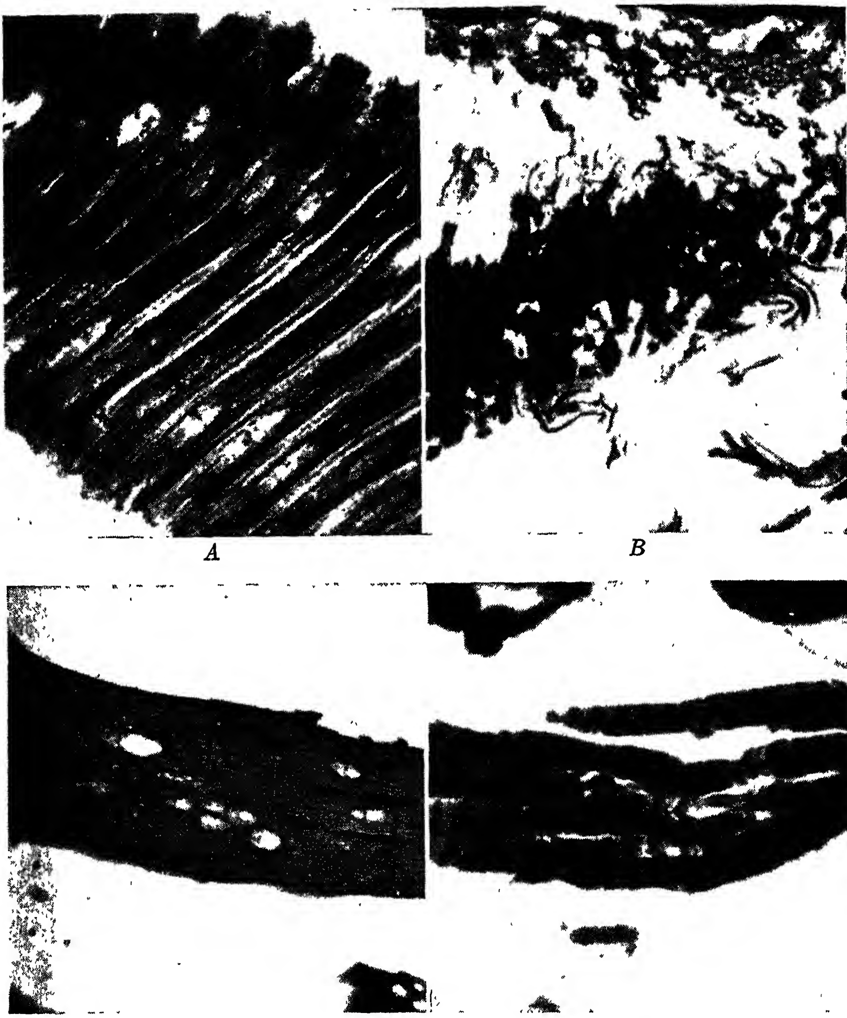

Fig. 13. Histological effects of pyrethrum compounds on tissues of the housefly. *A*. Section of the retinal layer of the compound eye as affected by pyrethrum. There are prominent vacuoles around the chromatin masses of the rhabdosome nuclei (at bottom of figure). Likewise, there are such vacuolate areas distally in the region of the pigment-cell nuclei. The nuclear membrane is most certainly broken down under such conditions. *B*. Severe changes in the nuclei of the anterior ganglionic mass as shown in section of the compound-eye system. The vacuolation and the obliteration of individual nuclei are a result of "Pyrin" treatment. *C*. Muscle of the head showing fenestration due to "Pyrin." This figure shows an appearance similar to that of the same muscle in a fly treated with pyrethrum alone, as in *E*. *D*. Normal muscle from the same region of the head as in *C*, *E*, and *F*.

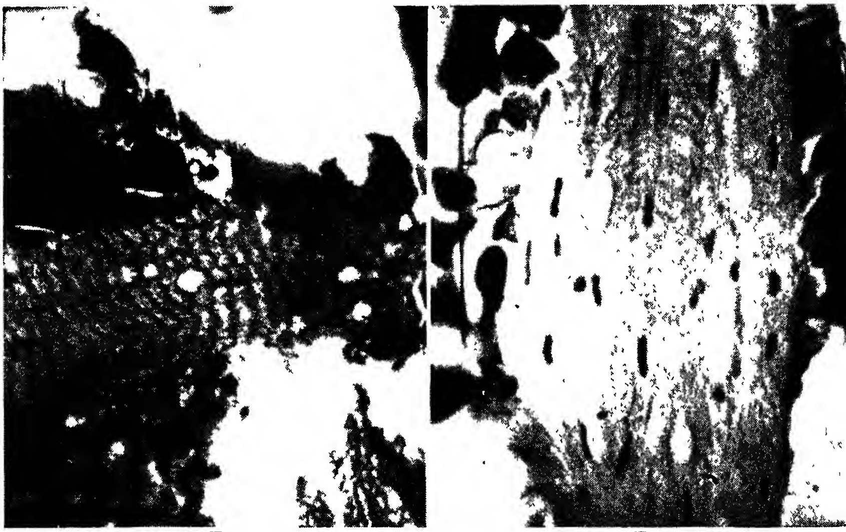
E F

Fig. 13 (*Continued*). *E*. Muscle of the head showing the very characteristic pyrethrum picture. *F*. Muscle as in the preceding figures, but showing the effect of the activator in the different character of the nuclei. (*A–F from Hartzell and Scudder, 1942; courtesy of A. Hartzell, Boyce Thompson Institute.*)

Working with the housefly, *Musca domestica* (Linn.), Hartzell and Scudder (1942) found that pyrethrum had a widespread clumping effect on the chromatin of the nuclei while the pyrethrum activator, isobutyl undecylene amide, appeared to cause a chromatolysis or dissolution of the chromatin. A combination of these two substances (as in "Pyrin") showed a histological picture that was a summation of the effects of both. Later Hartzell and Strong (1944) reported that the alkaloid piperine caused destruction of the fiber tracts and vacuolation of the nerve tissue of the brain of the housefly, but the widespread clumping effect of the nuclear chromatin characteristic for pyrethrum was not observed. The principal effect of sesamin and sesame oil on the nervous tissue of the housefly appears to be the vacuolation of the larger nerve cells of the brain (Hartzell and Wexler, 1946). Vacuolation of nerve tissue also occurs in the German cockroach, *Blattella germanica* (Linn.), poisoned with the gamma isomer of hexachlorocyclohexane (benzene hexachloride, or 666). This observation has been made by Srivastava (1948), who also noted an accumulation of free fat droplets in the neurophile mass. The pathological changes in nerve and other tissues induced by 666 could be inhibited by feeding the cockroaches a diet of inositol only during the early stages of its development. Such treatment also increases the resistance of the adults

to toxic doses of the poison. Feeding inositol to the adult insects has very slight protective effect.

With regard to pyrethrum, the conclusions reached by Richards and Cutkomp (1945) have, at least temporarily, thrown a somewhat different

Fig. 14. Longitudinal section of the mesobasi-sternal region of a German cockroach, *Blattella germanica* (Linn.), showing cuticle, hypodermis, fat cells, and mesothoracic ganglion (largest part of figure). The roach was poisoned with 0.33 milligrams of hexachlorocyclohexane (benzene hexachloride). Note vacuoles in the neurophile mass. (*Courtesy of A. S. Srivastava.*)

light upon most of the earlier work along these lines. These workers believe that the histological changes in nerve tissue poisoned by pyrethrum are similar to those produced by autolysis and may not be caused directly by the poison. They agree, however, that pyrethrum does have a selective action on nerve tissue. As seen with polarized light, they observed in cockroaches that pyrethrum first causes degeneration of the colloid of the axis cylinder and probably of the nerve cells also. The degeneration of the nerve sheaths occurs later (see also Richards, 1943). The appearance of vacuolation coincides with the time of breakdown of the lipoprotein

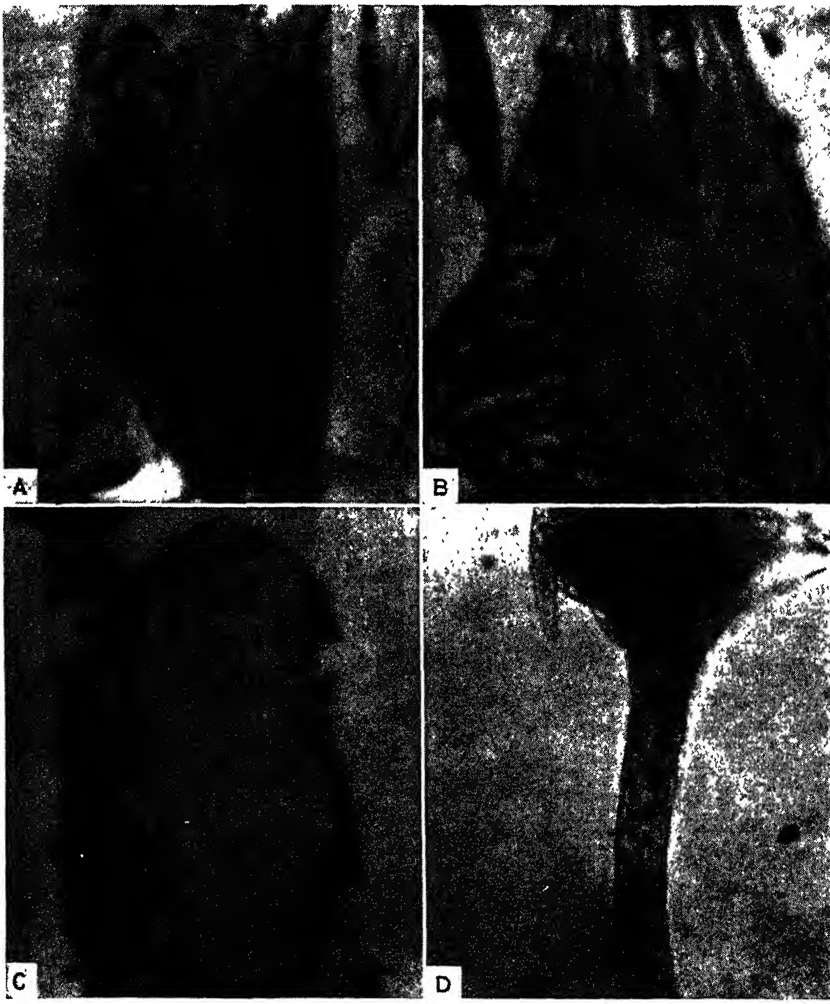

Fig. 15. Histopathological changes in the nervous tissue of poisoned insects (*Aëdes aegypti* (Linn.)). *A*. Stained longitudinal section of the fourth abdominal ganglion of mosquito larva dying from the effects of isoborneol thiocyanoacetate (active principle of "Thanite"). Drug injected into tracheal system. Note excessive vacuolation of fiber-tract region. Compare with *B*, which serves as a control for *A*. In *B* the tracheal system was injected with a "nontoxic" mineral oil. Tissue normal. *C*. Section of the subesophageal ganglion of a larva killed by tracheal injection of aniline, showing shrunken nuclei, which stain dark and solid and which separate abnormally. Fiber tract and intercellular spaces full of large holes, which correspond to the dissolution of birefringent particles produced by treatment with the drug. *D*. An unstained whole mount of an abdominal connective (nerve) of a larva killed by the tracheal injection of "Eugenol." Photographed by ordinary transmitted light to show rounded particles. (*From Richards and Cutkomp, 1945; courtesy of A. Glenn Richards.*)

sheaths and may be due to sheath products. All histological effects seen by these men in their experiments were subsequent to irreversible paralysis and were considered by them as post-mortem changes. They found these effects to be similar to those seen in the autolytic degeneration of nerves in saline. Thus, although the pyrethrum does kill the nerves, the resulting lesions may be due to causes other than the pyrethrum itself. Using a variety of other insecticides, they found that the nerves were paralyzed and presumably dead prior to the appearance of any abnormalities or lesions with the possible exception of chromatin clumping. No visible effects were obtained with DDT, although Hartzell (1945) reports some slight histopathological effects from this insecticide.

In summarizing the histopathological changes that take place in nerves of dead insects, Richards and Cutkomp point out that the first visible change, as seen by ordinary light, is that internally they become granular in appearance. The granularity is preceded by a loss of optical properties (using polarized light; see Richards, 1944) of the axis cylinder. This granularity is not often detectable in stained sections. Various kinds of large particles may appear in some cases and may occur either inside or outside the cells and fibers. Birefringent particles may be seen outside the fibers. Vacuoles may also occur either within the cells or between the cells and fibers, although the largest ones are found outside the cells. According to Richards and Cutkomp, the holes so often called "vacuoles" in insect histopathology do not necessarily represent vacuoles in the usual cytological sense. They represent the precipitation of tissue constituents around some particle or droplet which is subsequently dissolved during the preparation of the section. Thus in stained sections the "vacuoles" appears simply as holes with no indication as to their previous contents.

Such phenomena as shrinkage, opacity, and chromatin clumping appear to be best studied in unfixed nerve cords in saline, although chromatin clumping may also be observed in stained sections. Sectioned material is best for detecting variations in staining and for such degenerative changes as chromatolysis, cell and fiber separation, and cell and fiber degeneration.

Histopathology of Other Tissues in Poisoned Insects. In the discussion of the histopathology of the midgut wall we mentioned the fact that damage to the muscle layers may result in insects poisoned with arsenicals. Pyrethrum has been observed to form vacuoles within the muscle cells of larvae of *Corethra plumicornis* Fabr. (Krüger, 1931). After prolonged cramps, fissures appear in the muscle fibers together with a loss of their normal turgid appearance. Working with the housefly, Hartzell and Strong (1944) noticed that pyrethrum brought about fenestration of the

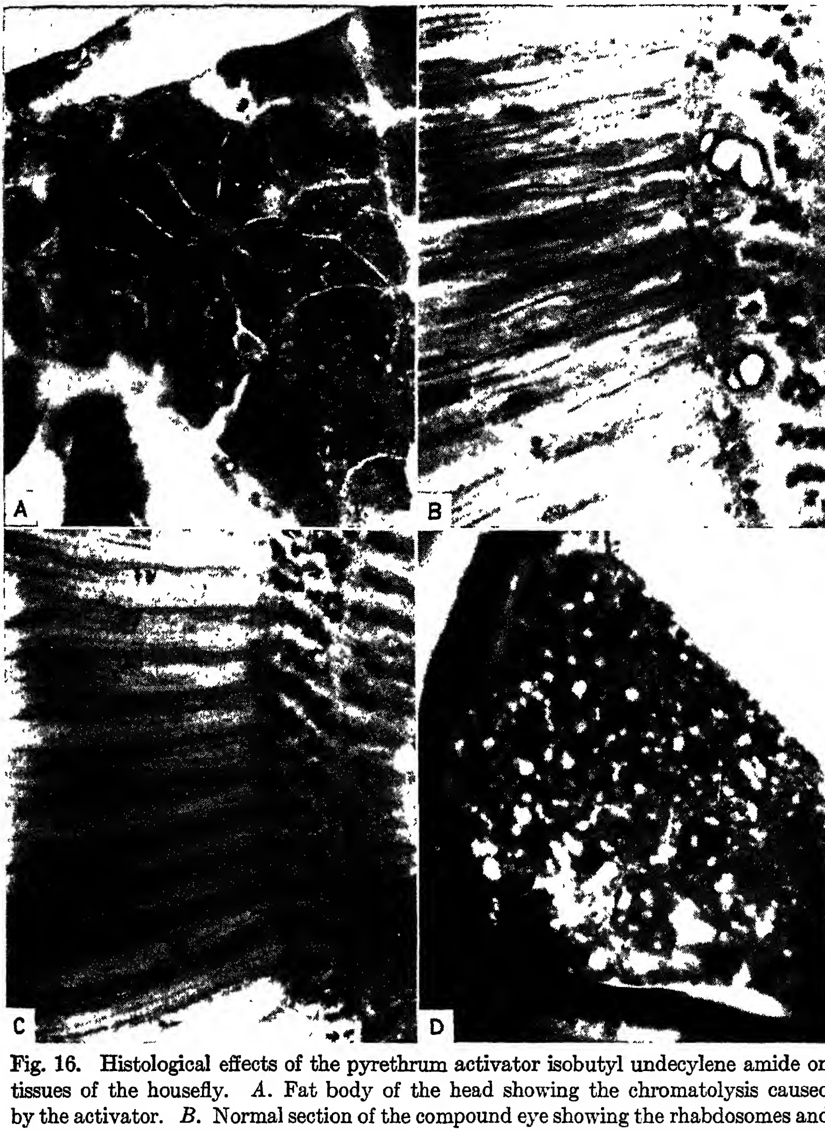

Fig. 16. Histological effects of the pyrethrum activator isobutyl undecylene amide on tissues of the housefly. *A*. Fat body of the head showing the chromatolysis caused by the activator. *B*. Normal section of the compound eye showing the rhabdosomes and the postretinal fiber layer, which is in turn underlain by the palisade cell layer, visible in the right hand third of the figure. *C*. Section of the compound eye showing the distorted angular nuclei of the cells that have invaded the postretinal fiber layer. The nuclei of the rhabdosomes show marked chromatolysis. The pathology is that of the activator. *D*. Fat body of the head showing the effect of "Pyrin." The nuclei are similar to those in *A*, which show effects of activator alone. *(From Hartzell and Scudder, 1942; courtesy of A. Hartzell, Boyce Thompson Institute.)*

cytoplasm, clumping of the nuclear chromatin, and loss of striations in the muscles, while piperine affected only an enlargement and accentuation of what is probably Krause's membrane. Sesamin and sesame oil affect the striated muscles of the housefly principally by the accentuation of the nodes and Krause's membrane. When sesamin and pyrethrum are combined, the typical effect is a clumping of the chromatin of the nuclei into rodlike masses, similar to that caused by pyrethrum alone (Hartzell and Wexler, 1946).

Fig. 17. Part of a longitudinal section of the fat body of a German cockroach, *Blattella germanica* (Linn.), poisoned with hexachlorocyclohexane (benzene hexachloride), and showing aggregated lipoidal globules in the fat cells. Stained by solutions that included osmic acid. (*Courtesy of A. S. Srivastava.*)

Hartzell and Scudder (1942) observed histopathological changes in several other tissues of houseflies poisoned with pyrethrum, including the fat body of the head region and parts of the compound eye. The fat body showed a separation of the cells and a clumping of the nuclear chromatin. Sections of the retinal layer of the compound eye showed prominent vacuoles around the chromatin masses of the rhabdosome nuclei. In the fat cells of cockroaches (*Blattella*) poisoned with hexachlorocyclohexane (benzene hexachloride) there is an aggregation of lipoidal droplets which are resistant to the action of xylol and neutral turpentine (Srivastava, 1948).

The histopathological effect of poisons on other tissues and systems in insects has been very inadequately studied, and practically no detailed information is available in the literature.

Natural Poisons

Although the injury of insects by compounded or synthesized chemical poisons is the most commonly known type, in noteworthy instances insects may be injured or killed through the ingestion of, or contact with, certain poisonous substances in nature. Such instances are noted much more frequently in the case of beneficial, commercially valuable insects, such as bees, but no doubt other insects are similarly affected.

As concerns the natural poisoning of bees, there are three principal types of such poisoning. They have been included by Butler (1943) in a series of conditions that he designates as types of bee paralysis, as follows: the poisonous-pollen type, the poisonous-nectar type, and the poisonous-honeydew type.

Poisonous-pollen Type. This type of poisoning has been reported chiefly from Europe, particularly in an area near Bettlach (hence the European term "Bettlach May-sickness"), and is caused by pollen of the wood-buttercup, *Ranunculus puberulus* Koch. This buttercup is visited by the bees only when no other forage, such as the dandelion and the cherry flower, is available. The poison substance may be anemonol or some closely related substance.

The poisoned bees tremble and cannot fly but twirl rapidly about or turn somersaults. Usually their bodies are held in a curved position with wings spread apart and the proboscis extended. The bees die soon after symptoms appear; experimentally they die from 3 to 5 days after feeding on the pollen.

This disease should not be confused with another condition that Butler (1943) designates as the "damaged pollen type" of paralysis, in which the affected bees have dilated abdomens and their colons are filled with ruptured pollen grains. Apparently pollen affected by low temperatures and frost is the cause of this ailment, since it usually manifests itself when a flying day follows a frosty night. Nor should either of these diseases be confused with the so-called "fungal poisoning" type of paralysis, a poisoning that is believed to arise from the toxicity of the spores of certain fungi (*Aspergillus*). The colons of such bees are often incidentally filled with pollen, and their abdomens are frequently distended. The muscles of the intestines are apparently paralyzed or damaged, making defecation difficult or impossible.

Poisonous-nectar Type. The number and kinds of plant nectars poisonous to insects have not been thoroughly investigated, but several are definitely known to be fatally poisonous to honeybees. In this country the nectar of both the California buckeye (*Aesculus californicus* Greene) and the spotted loco weed (*Astragalus lentiginosus* Douglas), among

others, have been incriminated, and in England there is some evidence that nectar from some varieties of cultivated rhododendrons is poisonous to bees. A number of additional plants have been suspected of being poisonous to bees, but definite proof of this is lacking.

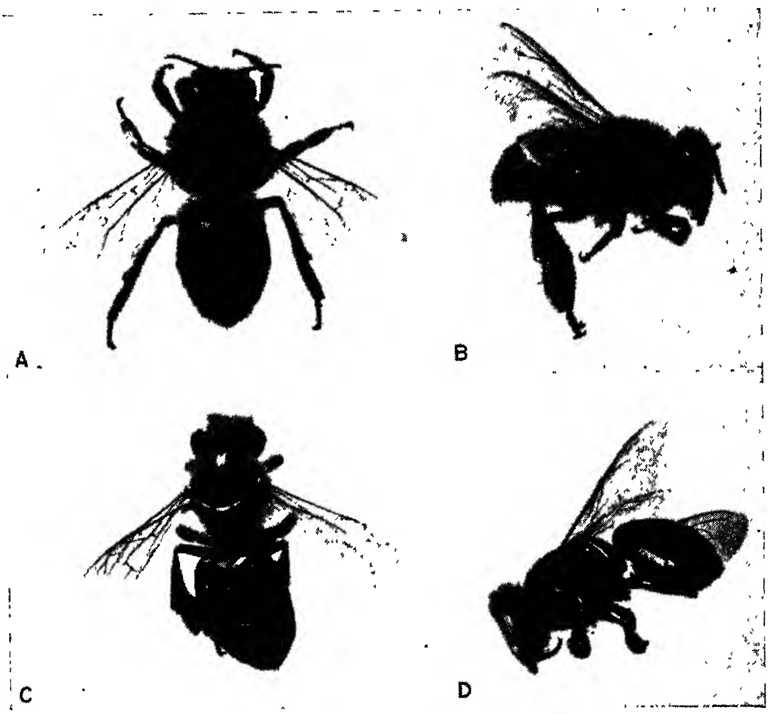

Fig. 18. Buckeye poisoning of the honeybee, *Apis mellifera* Linn. *A* and *B*. Normal adult worker bees showing the normal hairy covering. *C* and *D*. Poisoned bees from the hive, no longer able to fly. Such bees are nervous (shaky), distended with a foul material, and are picked bare of hair by the teasing of normal bees evidently trying to cause their departure from the hive. (*From Vansell, 1926.*)

In California the condition is known as "buckeye poisoning." It is particularly serious in certain parts of the state during years when there is a deficiency in topsoil moisture or when other plants fail to produce enough nectar to be more attractive to bees than the buckeye plant. California buckeye is found principally in the foothill zones around the Sacramento and San Joaquin valleys.

Buckeye honey, pollen, nectar, and sap may all affect bees severely. According to Vansell (1926), who made one of the first detailed reports on buckeye poisoning, not only field bees, but adult queens and drones, as well as the larvae and emerging young adults, are affected. In severe

cases the entire colony dies, leaving the hive filled with honey. The larvae that are not killed outright by the poison while being fed will pupate and emerge unless they are so greatly deformed that they are unable to do so. The newly emerged adults may appear with but four normal legs and unexpanded wings. Many are so weak that they die in the hives, from which they are carried out by the survivors and dumped in a pile in front of the entrance. Although the field bees seem to be affected less severely,

Fig. 19. Emerging honeybees deformed by buckeye poisoning. *A–D*. Deformities of legs and wings make these organs useless to the insects. Such individuals are pulled from the cells and removed from the hive by normal bees. Sometimes the legs are normal, but the wings never expand to be used in flight. (*From Vansell, 1926.*)

most of them eventually are unable to void their feces, and their bodies tremble or assume a "shaky" attitude. Their abdomens may or may not be swollen. According to Vansell, some bees become distended with a foul material "similar to a dysenteric condition." Such bees are picked bare of hair by the teasing of normal ones, which evidently try to cause the sick bees to leave the hive. Large numbers of dead bees are frequently found on the buckeye blossoms in the field or clustered together on foliage near the hive. The egg-laying power of the queen is drastically reduced. Even if the colony is removed from the buckeye location the effect of the poison continues as long as buckeye pollen remains in the combs.

An important contributary cause of the poisoning may be the sap that exudes from the punctures made in buckeye twigs and leaves by a small plant bug *Urbisea solani* Heid. and by *Gerhardialla delicatus* Uhl. This sap is collected by the hive bees, which may be poisoned by it.

Recommended control measures include the cutting of the buckeye

trees or the removal of buckeye honey stores and the relocation of the bees to pasturage where buckeye is scarce. Certain strains of hybrid bees seem to be resistant to the poison.

In Lyon County, Nevada, adult bees have been found to be poisoned while working the spotted loco. The bees become greatly weakened and may die. This condition becomes serious only in certain seasons.

Poisonous-honeydew Type. This particular type of poisoning is described by Butler (1943) as occurring in England and probably in Switzerland and in the Black Forest, where it is always associated with the conifer honeydew flow, usually spruce honeydew. After nights of rain or of heavy dew the mortality is lessened. Experimentally, Butler found the honeydew of a Homoptera on lime (*Tilia platyphylla* Scop.) to be extremely toxic to honeybees even when considerably diluted or after it had been filtered, boiled, or evaporated to dryness.

The bees affected by this type of poisoning first show agitation at the front of the hive. Later they become incapable of flight and, with wings more or less sprawled, crawl rapidly away in all directions from the hive. Morison's cell inclusions in the wall of the midgut may or may not be present.

There are some indications that the honeydew of aphids may be detrimental to lepidopterous insects (see Beirne, 1947), but it is questionable whether seasonal scarcities of Lepidoptera as a whole can be ascribed to this cause. Some authors have postulated that the honeydew favors the spread of disease among lepidopterous larvae or reduces the resistance of the larvae to disease. That such is actually the case, however, remains to be proved.

4. INJURIES DUE TO PARASITIZATION OR INFESTATION BY OTHER INSECTS OR ARACHNIDS

Either or both of two general types of injury may befall an insect that is parasitized by or infested with another arthropod:

1. Mechanical
   a. Destructive
   b. Irritating
2. Physiological
   a. Disruption or obstruction of normal physiological function
   b. Introduction of toxic substances

In other words, these injuries may be mechanical or physiological in character. If mechanical, they may be destructive (*i.e.*, the parasite may actually destroy the living tissue of the host), or they may simply be the type of injury that irritates or otherwise annoys the host (*i.e.*, certain mites may cling to the host insect without destroying any living tissue). From

one point of view these mechanical injuries could have been considered in our discussion on mechanical injuries at the beginning of this chapter. Since they are of a peculiar and more or less characteristic type, however, we have grouped them here along with the physiological injuries caused in insects by other insects and arachnids.

The type of mechanical destruction in any particular host will vary with the species of parasite concerned. A slight and usually inconsequential injury is done to the body wall of the host by those parasites which puncture the integument in ovipositing their eggs. Some ectoparasitic larvae puncture the skin of the host and imbibe the body fluids through the rupture thus formed. Endoparasites, during their early life, may simply lie within their hosts, surrounded by the blood or the serous fluid from which they gain their nutriment. Usually their activities eventually result in the death of the insects attacked, though this may take place after the parasites have left the bodies of the hosts. The partial or complete suppression of reproductive functions of parasitized adults frequently occurs. Occasionally an entomophagous insect so parasitizes its host (sometimes by what is known as "parasitic castration") that modifications of certain secondary sexual characters, such as the color pattern, result. In some endoparasitic Hymenoptera the embryo is surrounded by a cellular membrane which usually disintegrates either just before or just after the larva has assumed an independent existence. Its cells separate or adhere in small aggregations and become liberated in the body cavity of the host. Their function is nutritional, usually serving as food for the growing parasitic larva. In some parasites the body fluids of the host do not supply enough nutriment to supply the demands of growth, and the parasites therefore turn to the more solid tissues and organs as their source of food. To a certain extent the host suffers in proportion to the importance of the tissue or organ to the life of the insect. Usually the growing parasites restrict their feeding to nonvital structures of the host. This fact, together with the great tolerance insects have to parasitism, accounts for the fact that the visible effects of insect parasites upon their hosts are frequently slight and much less than one might be led to expect. Occasionally, however, vital organs of the parasitized insect are affected and the abnormal appearance of the host is quite distinct. Sometimes the injuries are somewhat indirect, such as when the small tracheal vessels are lacerated with the result that air is admitted directly into the body of the host.

Reactions of the Host. The reactions of the insect host and the damage it receives may logically be considered as pathological manifestations. The nature of these manifestations depends not only upon the parasite concerned but upon the kind and number of the tissues involved. Different tissues may be attacked by different parasites in the same host species. For

example, the larva of *Zenillia roseanae* B. & B. lives in the adipose tissue of the caterpillars of *Pyrausta nubilalis* Hbn., that of *Paraphorocera senilis* Meig. in the tracheal sheath, and that of *Angitia punctoria* Rom. floats free in the body cavity of the corn borer. On the other hand, one species of parasite may invade or destroy several kinds of its host's tissues.

The most common reaction of the host to an insect parasite is death. As a rule, the host dies soon after the parasite has left its body. There are

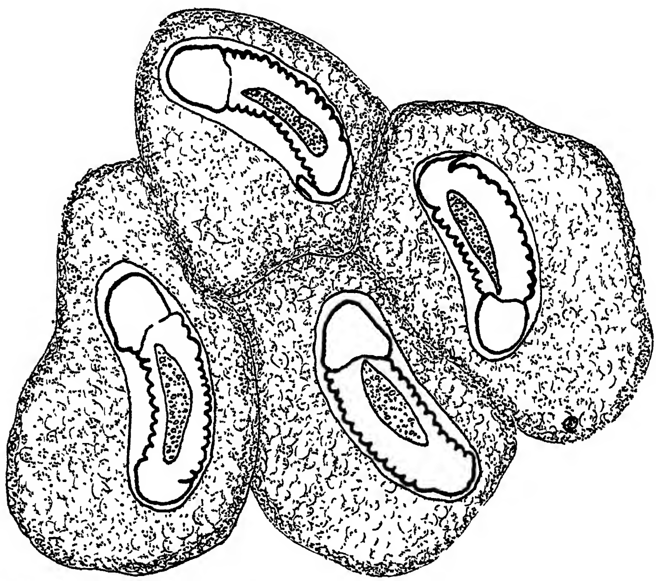

Fig. 20. Eggs of *Eulimnerium alkae* E. & S. in the body cavity of a corn-borer larva and surrounded by pseudocysts formed by the hemocytes of the host. Such pseudocysts represent a reaction of the host to parasitic invasion. (*Redrawn from Paillot, 1928.*)

exceptions to this, however, since cases are known in which the host survives, at least for a time, after the parasite leaves. It should be remembered that those parasites best adapted to their hosts do not kill their hosts or severely damage their vital tissues.

Before death ensues, however, the body of the host may react to the presence of the parasite in a number of ways. One of the more common of these reactions is that of phagocytosis. Certain hosts are capable of destroying the eggs oviposited in them by the adult parasites. This destruction appears to be due to an envelopment of the eggs by the blood cells of the host. For example, the eggs of *Eulimneria alkae* E. & S. and *Eulimneria crassifemur* (Thoms.) are destroyed apparently by this means in European-corn-borer larvae. Pseudocysts are thus formed which remain connected with the posterior part of the digestive channel of the

host. The cells that form these pseudocysts are uniform in type, having relatively large nuclei, and are morphologically identical with macronucleocytes (lymphocytes). The dead eggs are ensheathed in two distinct layers of these cells, the inner one of which is composed of round cells having distinct nuclei but indistinct borders. The outer layer consists of spindle-shaped, flattened cells, at times resembling fibers. When suspended in fresh blood, these fusiform cells regain their form as macronucleocytes. Although the parasite embryo at first develops normally, it is eventually killed when the cellular layer has reached its maximum thickness. Such phagocytic action differs in various host larvae, but it is alike on eggs of different parentage deposited in the body cavity of the same host (Paillot, 1928). Some investigators have reported that the eggs of certain parasites are digested in the midst of these phagocytic cellular masses occurring in some hosts. Such is not the case in the instance cited above.

Parasitic larvae may be phagocytosed in a manner similar to that just indicated for the eggs. As reported by Strickland (1930), the eggs of a tachinid, *Gonia*, hatch in the mesenteron of the host; the larvae bore through the peritrophic membrane and the mesenteric wall into the body cavity, and then into the supraesophageal ganglion. If the larvae are successful in entering the ganglion and feeding there a little, they usually escape into the body cavity without being attacked by phagocytes. On the other hand, unfed larvae that have not entered a ganglion are almost invariably surrounded by phagocytes which ultimately bring about their death.

It should be pointed out that sometimes the host reacts in a way that actually benefits or protects the parasite and enables it to develop more satisfactorily. An example of this is the ingrowth of the body wall of the host around certain tachinid parasites during the latter's development.

The reactions of the host may be visible externally as well as internally. Usually the reactions thus seen are those concerned with the size and function of the various anatomical parts of the host. Reduction in the size of the host is a common result of parasitization even when the host survives the parasitic attack. Other abnormal forms frequently ensue from parasitism, and occasionally these have been given descriptive names. In reviewing the different morphological phases known to occur among ants, Wheeler (1926) divides them into two main groups, the normal and the pathological phases. Most of the pathological forms arise from some type of parasitism. Thus he describes the "phthisaner" phase as a pupal male which in its larval or semipupal state has had its body fluids partly extracted by an *Orasema* larva. This male is unable to pass on to the adult stage. The wings are suppressed and the legs, head, thorax, and antennae remain abortive. The "phthisogyne" arises from a female

larva under the same conditions as the phthisaner and differs from the typical female in essentially the same characters. The same is true for the "phthisergate" phase which is a pupal worker having a modification of characters similar to those just described. The "pseudogyne" is a workerlike form with an enlarged mesonotum, and it occasionally has traces of other thoracic sclerites of the female. This form is produced by the presence of Lomechusine beetles in the colony. The "mermithergate" phase is an enlarged worker resulting from parasitization by *Mermis* worms. Its thorax approaches that of the female in size, and it has minute ocelli in its head. Other pathological phases occurring in ants Wheeler has termed "pterergate" (a worker or soldier with vestiges of wings), "gynandromorph" (an anomalous individual in which male and female characters are combined), and "ergatandromorph" (similar to the last but having worker instead of female characters combined with those of the male).

Not all the effects of insect parasites on their hosts are detrimental. Cases in which the development of insects has been accelerated by such parasitization are known (see Varley and Butler, 1933).

Acarine Disease, or Isle of Wight Disease. Sometimes the parasitism or infestation of one arthropod by another is of a contagious nature and produces serious enough consequences to be considered a disease. Such an instance is that of the Isle of Wight disease or, as designated by some, the acarine disease of honeybees (*Apis mellifera* Linn.). The latter name denotes the fact that the disease results from an infestation with a mite (order Acarina).

The disease was probably first observed in the southeastern part of the Isle of Wight in 1904, from which it quickly spread over the island so that by 1908 most of the original bee stocks had perished. During the years following, it spread rapidly throughout Great Britain, though it had probably existed in England prior to this time. Following 1920 it was reported from countries on the European mainland and from South Africa. It is not known for certain to occur in the United States.

The Isle of Wight disease was at first thought to be due to an infectious microorganism such as the microsporidian *Nosema apis*. Rennie and his coworkers, however, began to doubt this etiology about 1916, and in 1921 they showed the ailment to be caused by a mite which they named *Tarsonemus woodi*. This mite was subsequently transferred by Hirst to another genus as *Acarapis woodi* (Rennie).

The mites enter the body of the honeybee through the first thoracic spiracles on either side of the body. From this location they obtain their nourishment from the blood of their host. The tracheal trunks become spotted with feces that color the normally white walls a brown or black. The mites may become so numerous as to plug the tracheae almost com-

pletely, making the passage of air practically impossible. This deprivation of air is believed to affect the aeration of vital tissues to which the tracheae lead. According to Anderson (1928), mechanical injury is inflicted on the tissues adjoining the infested tracheae, including the thoracic salivary glands, the indirect flight muscles, and the large nerves passing to the base of the wings. It is assumed that such injury would cause a diseased condition in the punctured muscle and nervous tissue and that paralytic

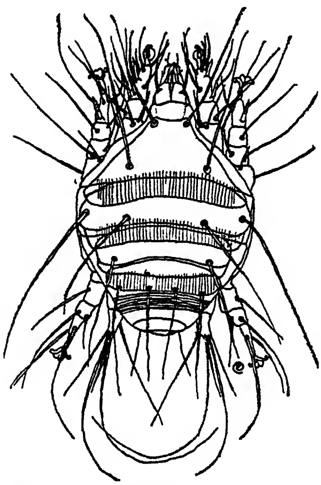

Fig. 21. Female of the mite *Acarapis woodi* (Rennie), the cause of acarine disease in the honeybee. (*Redrawn from Hirst,* 1922.)

Fig. 22. Trachea from thorax of honeybee infested with *Acarapis woodi* (Rennie). The mites may be seen through the tracheal wall. (*Photograph by Rennie; courtesy of C. E. Burnside.*)

symptoms would probably result. The fact that the adjacent flight muscles are affected may possibly account in part for the inability of affected bees to fly. This is the predominating symptom of the disease. The bees crawl about sluggishly on the ground with "dislocated" wings and are unable to fly. It is common to see them fall from the alighting board to the ground. Such bees may, in cool weather, gather in little clusters on the ground or grass in front of the hive and die of hunger or exposure. In the spring badly infested bees are usually heavily laden with feces, probably because of their inability to fly during winter (Phillips, 1923). (See also Dade, 1948.)

In addition to the puncturing of the tracheal walls as the mites feed on the blood of the host and the stopping of air circulation, there is some evidence that pathological changes, other than those already mentioned, occur in the adjacent tissues (White, 1921). Furthermore, it is possible

that the mites may produce a toxic substance that is absorbed by the host.

Attempts to control the infestation by the application of chemicals or insecticides has not met with significant success. The so-called "Frow treatment," however, has been popular in some quarters. This consists of the use of the vapors from a combination of nitrobenzene, safrol oil, and gasoline. Some beekeepers have obtained equally good results with nitrobenzene alone. Since the disease is a contagious one, being spread by the mites crawling out of one host onto and into another and by migrating females, the most effective means of control is the elimination of infested bees. Large numbers of young bees should be reared to take their places, thus reducing the infestation. Good management and effective swarm-control measures are also important factors in reducing the amount of infestation.

Physiological Injuries in Parasitized Insects. Like the mechanical injuries that occur in an insect parasitized by another insect, the type of physiological injury varies with the species of host and parasite concerned. In nearly all cases of the latter type of injury, however, there is either an obstruction or prevention of normal physiological function on the part of the host or there is an introduction of toxic substances that directly affect the physiology and metabolism of the host.

When an insect's tissue is damaged or destroyed, of course its function is also impaired or destroyed. When the lymph or stored food reserves of the host are diminished or depleted by an internal parasite the physiology or metabolism concerned is correspondingly altered. Sometimes the parasite may not actually destroy much of the tissue but does usurp some of the benefits of the tissue's functioning. For instance, this occurs when parasites attach themselves to the host's tracheae primarily to gain an air supply for themselves. This type of parasitization, incidentally, frequently exhibits external changes in the insect that simulate certain of the infectious diseases. Insect parasites attached to the tracheae may, for example, cause a mottling or necrosis to appear in the integument about the spiracular opening of the host.

It is conceivable that endoparasites may liberate toxic substances from their bodies as they develop in the host. In most endoparasitic larvae, however, there is no passage between the midgut and the exterior; hence in these cases the host is not contaminated by the intestinal waste products of the parasite.

A more common intoxication occurs when an adult parasite paralyzes the larval host by injecting it with a toxic fluid before it deposits its egg. For example, *Habrobracon brevicornis* Wesm. does this when it oviposits on the corn borer, *Pyrausta nubilalis* Hbn.

Analogous to this is the well-known phenomenon associated with the solitary Hymenoptera which use their venom for paralyzing their prey—usually caterpillars or spiders. In such cases the victims remain paralyzed for months, although the heart continues to beat. In at least one case

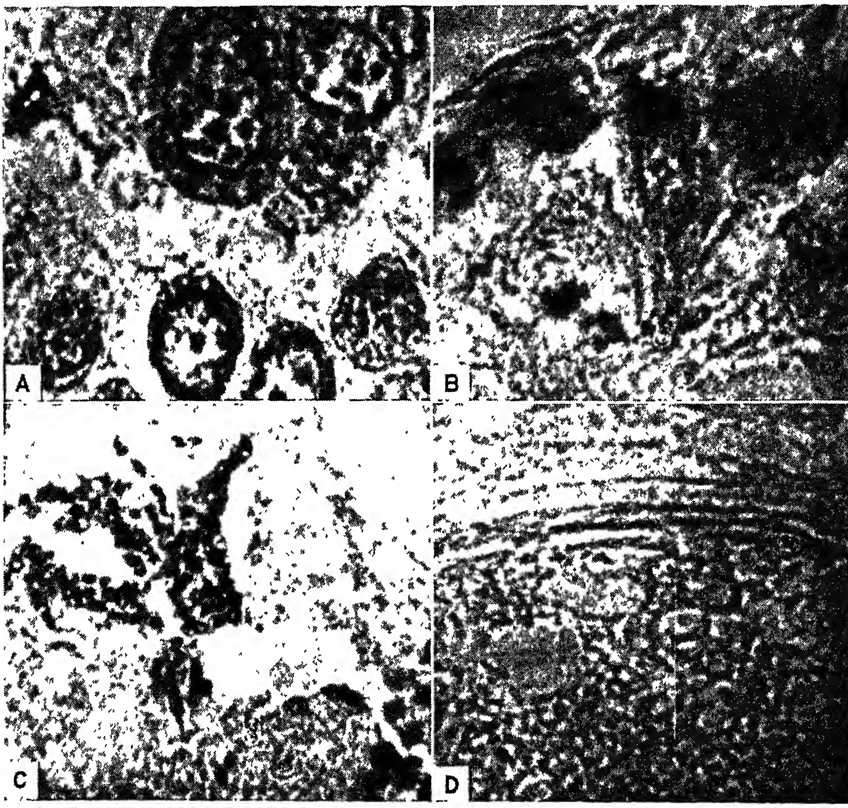

Fig. 23. Histopathological changes in the nerve tissue of a cicada, *Tibicen pruinosa* Say, after paralysis by the killer wasp, *Sphecius speciosus* Dru. Cross sections stained with toluidine blue. *A*. Brain of paralyzed cicada; note marked tigrolysis and vacuolization. *B*. Brain of cicada killed by decapitation. *C*. Thoracic ganglion of paralyzed cicada; note disintegration and vacuoles. *D*. Thoracic ganglion from specimen killed by decapitation. (*From Hartzell, 1935; Boyce Thompson Institute.*)

the histopathology of the nerve lesions of a venom-paralyzed insect has been studied. Hartzell (1935) has reported that adult cicadas (*Tibicen pruinosa* Say) paralyzed by the sting of the killer-wasp (*Sphecius speciosus* Dru.) showed nerve lesions in the main parts of the central nervous system. In many respects the lesions were similar to those produced in the nerves of insects killed with triorthocresyl phosphate and with the pyrethrins.

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CHAPTER 3

DISEASES OF NUTRITION AND METABOLISM

Among the noninfectious ailments or diseases to which insects are subject are those which arise from faulty nutrition and from deranged physiology or metabolism. These factors are so interrelated that in most cases the disturbance of one automatically upsets the proper functioning of the other; *i.e.*, a faulty nutrition frequently brings about an upset or deranged metabolism, and vice versa. For the sake of convenience in discussing them we shall, however, treat these factors separately, although this is largely an arbitrary matter. Furthermore, it will not be our intention in this chapter to give an exhaustive treatment of the subject. Only the principal pathological features will be mentioned and these only briefly and for the purpose of assisting in the orientation of the student with respect to the various types of pathologies occurring in insects.

NUTRITIONAL DISEASES

By and large, nutritional diseases are those conditions which arise as a result of general nutritional deficiencies or from the lack of some particular food constituent in the diet. Such absence of critical nutrients usually shows itself by its effect on the growth, development, and reproductive capacity of the insect. The deficiencies may take a variety of forms, the more common of which will be discussed briefly in the following paragraphs.

Lack of Food. Starvation, as we usually think of it, refers to the condition or the suffering arising in an animal from the deprivation of food against its will or desire. This includes those cases in which monophagous insects will deliberately starve to death in the absence of their proper food plant; and, according to Brues (1946), most oligophagous species with a highly restricted diet will do the same. Like most animals, insects are usually unable to withstand a complete lack of food for more than a few days. To be sure, a few insects such as certain moths and flies are devoid of functional mouthparts and cannot feed in the adult stage, but these forms are relatively short-lived. In those insects which undergo complete metamorphosis, the pupal stage does not feed, but the metabolic processes depend upon the reserve of food substances stored in the developing insect's body. Insects deprived of food may survive for periods ranging from a few days or weeks to several years. Even the larval stages of

certain beetles are able to withstand starvation for long periods of time. For example, the larva of *Trichodectes ornatus* LeConte has been known to survive for 5 years without food. Other instances of marked longevity in the absence of food have been reported.

A lack in the over-all quantity of food usually eaten by an insect may also be considered as a type of starvation. Such a deficiency may affect the final size of an insect's body, although this variation actually may not be very great. The larvae of certain wasps, for example, are each fed a single large spider by the mother. These spiders vary greatly in size and accordingly the fully developed wasp will also vary considerably. Similarly, certain entomophagous insects will show variations in size corresponding to the relative sizes of their hosts.

In most insects starvation begins with a consumption of the carbohydrates present in the tissues. This is indicated by the rapid drop in the amount of glycogen present. Some insects consume their proteins extensively while others make but slight use of their tissue proteins. The principal reserve substance utilized by insects is fat. From 50 to 90 per cent of the fat is used up by most insects before they die of starvation.

It is important that the insect pathologist be aware of the possibility that dead insects found in the field or submitted to him for examination and diagnosis may have succumbed to the effects of starvation rather than to those of poisons or pathogenic microorganisms. A starved insect can frequently be detected on close examination by a "withering" of its tissues and by the depletion of the reserve food supplies, particularly the fat body.

Lack of and Surplus of Water. Certain of the moisture requirements of insects have already been discussed, in Chap. 2, and it has been explained that the pathology of insects affected by drought or lack of water is essentially that of dehydration.

As pointed out by Wigglesworth (1939), the amount of water needed in the food depends upon the rate at which it is lost from the body; and this depends, on the one hand, upon the properties of the cuticle, respiratory system, and excretory system, and on the other, upon the drying power of the air. Insects that produce liquid excrement, such as the honeybee or muscid flies, must drink frequently if they are to survive; whereas food-product insects, such as the mealworm and insects living in deserts and other arid regions, extract almost all water from their excrement and can live on very dry substances. A great deal of the water required by these insects is produced as a by-product of their own metabolism. This metabolic water is gained when the carbohydrates in the body are oxidized to carbon dioxide and water. Such insects also have a greater ratio of bound water to free water in their tissues which makes them less susceptible to processes of evaporation.

Many insects are able to survive over long periods of time without imbibing fluids. An instance is recorded in which the preimaginal encysted stage of certain scale insects has been known to survive for 17 years without taking water.

There have been reports in which a dysenteric condition of honeybees was thought to be caused by the imbibing of too much water, but this condition is now thought to be due to other causes.

In the case of certain lepidopterous larvae (*e.g.*, the silkworm, the nun-moth caterpillar, etc.), a surplus of water on their food has been considered as a predisposing cause to certain dysenteries or flacheries. It is assumed by some that when plant foods, for example, stand for a long time in the rain or are held too long in water (as is frequently done to keep the food fresh), an injury to the plant protoplasm results along with an increase in the acidity of the leaves. When the caterpiller eats such leaves, the strong alkalinity of its digestive tract presumably becomes decreased and the insect's metabolism is upset, making it more susceptible to microbial invasion.

A radical change in the reaction of the gut contents may not be the only reason for these conditions brought about by a surplus of water. It may simply be a case of there being too much water in the leaves of the plant for the proper handling of the food in the gut of the insect. Microscopic examination of the gut contents will show undigested small particles of leaves floating about, indicating that the digestive functions have been impaired. The excrement of such insects, normally compact and blackish, becomes green and soft, and then liquid. If at this point the caterpillars are fed dry food, they usually recover without difficulty. If, however, the condition is allowed to progress, it becomes much worse, the bacteria in the gut multiply rapidly, the excrement becomes brown, slimy, and stringy, and the insect succumbs. If the wet leaves fed to the caterpillars are already in a decomposing state, an abnormal number of bacteria are introduced, and the disease is serious from the outset. Conditions brought about by the ingestion of foods holding an excess of water have been called "intestinal catarrhs" by some authors.

Lack of Organic Food Substances. The lack of organic food substances, such as proteins and carbohydrates, may affect the growth, reproduction, and energy production of the insect. It is possible for an insect to have enough food for energy production but not for growth. Cockroaches on a nitrogen-free diet are able to maintain their body weight for many months, and pure cellulose will enable termites to live for fairly long periods; but such diets do not ensure growth, for which insects must have a source of organic nitrogen, sulfur, phosphorus, and salts.

A deficiency of organic food substances may show itself in ways other

than that of a shorter life. On a diet very low in proteins the production of venom required by bees for the sting is reduced, and the insects become more docile. Certain blowflies have been found completely incapable of producing fertile eggs unless after emergence they receive an adequate amount of protein in their diet. In the case of social ants, bees, and wasps, the production of the infertile worker caste is believed to depend upon differences in the quantity or quality of food ingested during larval growth.

The lack of nitrogenous food has been cited as the cause of one type of paralysis in the honeybee, *Apis mellifera* Linn. (see Butler, 1943). Adult bees, especially nurse bees, that do not have enough nitrogenous material in their food (pollen) usually draw on the nitrogenous reserves of their own bodies. Most of this nitrogen is taken from the integument of the insect, the chitin becoming so brittle that the bees readily lose their hair, and even their wings may break off. The deficiency is most frequently noticed in hives that have been deprived of a store of pollen for a long period of time. It may be treated by supplying the colony with combs containing pollen. Soybean flour paste, which is readily digested and assimilated by the honeybee, may serve as a substitute source of nitrogen.

Incidentally, beekeepers in certain parts of the United States have, in the past, believed that the ingestion of pollen from the winter honey stores is at least partly responsible for a dysentery among bees. Present indications are that the eating of excess pollen is not necessarily conducive to dysentery. Pollen grains ingested by bees pass rather rapidly through the alimentary tract, and by the time they reach the hindgut most of them are empty and some may be collapsed, indicating their adequate digestion.

The absence of carbohydrates in the diet may be the cause of shortened life or depleted energy of an insect, since this organic foodstuff serves as an excellent source of energy for many insects. Some insects, such as the honeybee, can live four to seven times as long on sugars as when given pure water, and certain flies (*Calliphora*) will live 1 to 2 months on sugar water but will die in 2 or 3 days if given water alone.

Lack of Mineral Elements. The lack of certain minerals may be a factor in the limited growth of insects. The essential mineral elements appear to be potassium, phosphorus, and sodium. The low chloride content of water can be a limiting factor in the growth of mosquito larvae, and the lack of calcium may have a deleterious effect on these insects. Drosophila-fly larvae have been reared in the apparent absence of sodium or calcium, but potassium and magnesium seem desirable for their growth.

The deleterious effects of the lack of salts and minerals in the diet of insects is, to say the least, very inadequately known.

Lack of Vitamins and Accessory Food Substances. The details of the pathology in insects deprived of their required vitamins have not been

well clarified. The best criteria of a vitamin deficiency in an insect so far offered are a shortened life span, decreased egg production, and certain types of decreased activity.

It should be remembered that insects vary greatly as to their vitamin requirements. In fact, some insects apparently do not need certain of the vitamins in their diet. Thus *Drosophila* apparently needs no sources of vitamins A, C, and D. *Tribolium* requires no C or D vitamins, and Wollman has been able to rear cockroaches (*Blattella*) for 15 years on a diet free from vitamin C. On the other hand, most insects seem to require certain of the B vitamins, although some workers believe that neither vitamins B_1 nor B_2 are needed by drosophila flies but that some factor found in yeasts is.

As reviewed by Trager (1947), recent work indicates that most species of insects appear to require only one fat-soluble accessory growth factor, cholesterol. Although cholesterol can be replaced by certain other related sterols, it cannot be replaced by sterols of the vitamin D group. Water-soluble growth factors for insects appear to be identical with the water-soluble vitamins of the B group required by vertebrates.

The source of some vitamins for many insects may be certain of the microorganisms intimately associated with them. Some insects may be raised germ-free but only if they are provided with the necessary accessory factors that in nature are supplied by microorganisms. Both extracellular and intracellular microorganisms may assume this role. It has been shown in the case of the drugstore beetle (*Stegobium paniceum* (L.)), for example, that intracellular yeastlike organisms supply this insect with vitamins of the B group. When separated from their vitamin-producing microorganisms, the insects die. In a later chapter we shall have occasion to mention again the role of microorganisms in the nutrition of insects.

DISEASES CAUSED BY DERANGED PHYSIOLOGY AND METABOLISM

In the complexity of all the physical and chemical processes that have to do with the production and maintenance of the organized living substance of an animal, even one as small as an insect, many abnormalities and dysfunctions are likely to occur. In the case of insects, as with that of most other invertebrates, so little is known on this subject as to make any discussion of it a mere glimmer of what might actually exist. The very abundance of insects prevents most entomologists from being very much interested in the metabolic difficulties of any particular individual specimen. When physiological disturbances show up in large numbers of insects at one time they are more likely to be noticed and investigated. Even so, few such instances have been reported.

It is conceivable that any of the various types of metabolic activity ordinarily functioning in the body of an insect may become deranged or abnormally affected in some way. Especially is this likely to be the case with the following:

1. Fat metabolism
2. Carbohydrate metabolism
3. Protein metabolism
4. Respiratory metabolism
5. Hormone metabolism
6. Pigment metabolism
7. The metabolism and physiological processes important in the production of certain chemical products of insects (silk, lac, venoms, wax, scents, etc.)
8. The physiological or metabolic processes of an insect that may in general become deranged making the exact cause of the dysfunction practically impossible to determine (*e.g.*, see Palm, 1948, page 65)

The known causes of deranged metabolism are many and varied. A common cause, of course, is infection, but we are concerned here only with noninfectious conditions. Accordingly, one might assume that any of the chemical or physical factors so far discussed in this and the preceding chapter as adversely affecting the life of an insect at the same time almost invariably upsets some physiological balance concerned in its metabolism.

Sometimes the derangement of an insect's metabolism is of such a definite and characteristic type that it constitutes a discernible disease. In such a case, not only are the symptoms fairly characteristic and uniform for the disease but the pathology is usually of a distinctive nature. The amicrobic dysenteries of the silkworm (*Bombyx mori* Linn.) exemplify this sort of condition. Rather general discussions of this type of silkworm disorder may be found in publications by Paillot (1930) and Ishikawa (1936). Only the principal features of some of them can be mentioned here.

Flaccidiform Dysentery. *La dysenterie flaccidiforme* is the name used by Paillot (1930) to designate a condition in *Bombyx mori* Linn. arising from certain indefinite metabolic disturbances occurring during the time the silkworms are molting. It may be considered an accident of breeding in that, when the rearing conditions are markedly changed at the end of the molting period (particularly the third and fourth molts), the larvae are unable to survive the molting crisis. In a general way the symptoms are similar to those of flacherie and gattine. The larvae may appear slightly swollen and lethargic; they have a poor appetite and are diarrheic. The condition is in no sense contagious, and only a part of each group of larvae succumbs. The microbial flora of the intestinal tract is remarkably scant, and no microorganism has been found to which the cause of the malady could be attributed.

In 1925 Paillot observed a typical outbreak of flaccidiform dysentery in a colony of silkworms in the community of Vans in Basse-Ardèche, France. Mortality was fairly high at the end of the fourth instar. The larvae died in great numbers, presenting all the symptoms of true flacherie. The bodies, scattered about at random, blackened rapidly but did not give off the disagreeable odor so characteristic of flacherie (in which a virus and *Bacillus bombycis* are involved). Examination of the intestinal flora failed to reveal any microbial cause of the disease. All the conditions under which the insects were being reared seemed to be in perfect order. Up to the

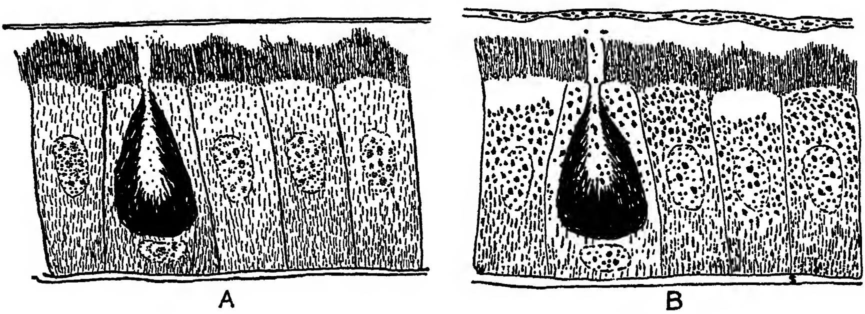

Fig. 24. Flaccidiform dysentery in the silkworm. *A*. Diagrammatic cross section of a portion of the midgut epithelium of a normal healthy silkworm. *B*. Midgut epithelium of a silkworm suffering from flaccidiform dysentery. Note pathological changes as described in text.

third molting period the larvae had been reared in a heated kitchen. At the end of this molting period some of the larvae were carried to another room and some were left behind in the kitchen. Those left in the kitchen remained healthy and normal in every respect. Some of those transferred to the other room, however, showed signs of illness, and by the end of the fourth molting period the mortality was extremely high. Both groups of insects had received the same food at the same time; the only difference was in the change of rearing conditions. The return to active larval life after a molt is a very critical period in the life of the silkworm, and apparently a change of conditions can affect the ability of the larva to overcome this crisis.

The histopathology of this disease is very interesting in that the use of ordinary methods of fixation and staining does not reveal any significant changes. On the other hand, if mitochondrial methods are used, slight histopathological alterations are noted in the protoplasm of the intestinal epithelial cells. In normal silkworms the mitochondria are arranged in elongated chondriocontes (masses of rod-shaped mitochondria) parallel to the long axis of the cell. In the diseased insects the diameter of the mitochondria is increased and some of the filaments are fragmented giving

rise to chains of rounded or spindle-shaped granules. In the region that borders the intestinal lumen the cells may appear to be deprived of all mitochondria, or fuchsinophilic blocks or masses may accumulate in this part of the cell. The true value and significance of such mitochondrial changes as these are not entirely clear, since similar alterations are known to occur in tissues under other conditions. The nuclei appear normal; but the nucleoli, instead of being fuchsinophilic, become basophilic, staining at about the same density as the chromatin granules. These changes are noted particularly in the epithelium of the midgut where they begin at the posterior end and progress to the anterior end. The cytoplasmic and nuclear lesions are accompanied by more or less active cellular destruction, resulting in a considerable thickening of the peritrophic membrane which appears spotted with fuchsinophilic granulations of a mitochondrial origin. The secretory processes of the epithelium also appear to be affected.

The prevention and control of flaccidiform dysentery are brought about principally by the use of rational methods of silkworm rearing. Among the most important factors to be considered Paillot mentions the following: (1) incubators and rearing rooms should be well regulated as to temperature and humidity; (2) the young larvae should be removed at a maximum of 3 days; (3) larvae of the same brood should all be of the same instar; (4) the number of feedings should be proportioned to the surrounding temperature; (5) the external conditions should not be changed during the time of molting; (6) strict sanitary conditions for both surroundings and food should be maintained at all times; (7) the larvae should be well spread out and given plenty of air.

Dysentery Associated with the Spinning Mill (Filature). On rather rare occasions this type of dysentery has been observed affecting silkworms in France. It is thought to be caused by the presence in the rearing rooms of abnormal amounts of the dust arising from the spinning operations. The machines that unwind the silk from the cocoons are commonly surrounded with certain silk wastes, debris, and dust, all of which are easily disturbed and carried by air currents. When growing silkworms are housed nearby or in adjoining rooms these waste dusts occasionally cause the insects to become ill.

Upon feeding silkworms mulberry leaves soiled with the dust formed in the room where the cocoons are handled, Paillot (1930) observed them to show a marked repulsion for such nourishment and to accept it only in very small quantities. Within 24 hours after the ingestion of the soiled leaves the first signs of diarrhea were noted. The same symptoms resulted when the larvae were fed products of their own excretion or secretion, *e.g.*, the silk waste in the form of the silk floss. When this silk waste is treated

in a drying oven or exposed to steam it loses its toxic properties. When it is extracted with ether and the solvent is evaporated, a brown waxy residue is obtained that has the characteristic odor of silk. Silkworms raised in the immediate neighborhood of this material are affected with a loss of appetite and some symptoms of diarrhea. Accordingly, it has been assumed that this condition is actually the result of the action of certain poisons that accompany the wastes and dejecta of the larvae. The somewhat volatile fluid that the insect secretes before it begins spinning

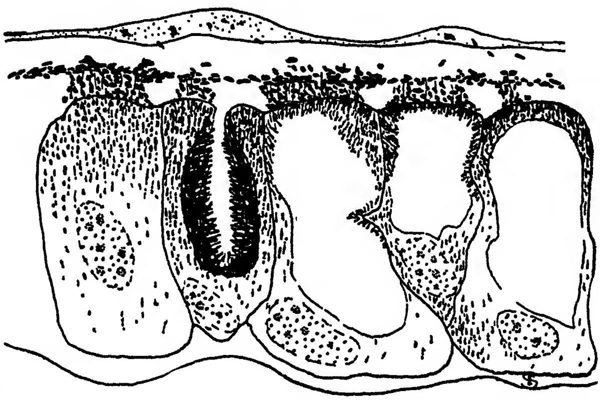

Fig. 25. Diagrammatic cross section of midgut epithelium of a silkworm suffering from dysentery associated with the filature. (Normal aspect may be seen in Fig. 24*A*.) Note thickening of peritrophic membrane, disintegration of the striated border, vacuolization of cytoplasm, and alteration of mitochondria.

its cocoon seems to be particularly concerned. This fluid apparently may act from a distance, and the action becomes more marked as it is prolonged.

Histopathologically, the principal lesions are found in the central portion of the midgut epithelium. The external border of the gut wall appears broken up, and one finds large vacuoles in the cytoplasm of the epithelial cells. In places, the peritrophic membrane is very much thickened. The striated border of the epithelium is condensed into a fuchsinophilic mass that separates from the cells. The mitochondria are granular and are irregularly distributed in the cell, frequently accumulating at its distal end.

Thus it is seen that this peculiar condition in the silkworm is brought about by the dust from the filatures, the ingestion of which causes marked alterations of the epithelial wall of the midgut. The action of the dust from the filature is not of a mechanical nature, since dusts from other sources have no such effect on the silkworm. The diarrhea, which constitutes the only external symptom of the disease, results from the great modification of intestinal secretory processes. It is not unlikely that the

toxic action here described also increases the susceptibility of the silkworm to the various microbial diseases to which it is subject.

Methods of prevention and control are essentially those which promote sanitation and extreme cleanliness. The insect should be in a location sheltered from the dusts of the filature. Dry sweeping should be avoided. Cleaning should be accomplished with a damp cloth, and the refuse collected should be removed to a far-distant place.

Pseudoflacherie. Another peculiar condition that on rare occasions is found affecting the silkworm, *Bombyx mori* Linn., is that which Paillot (1930) has designated as pseudoflacherie. The true cause of the disease is not known. Although the intestinal contents of the silkworms are rich in bacteria, the latter do not appear to have anything to do with the etiology of the disease.

In their external appearance the affected silkworms are similar to normal ones. Two noticeable characteristics are the almost complete immobility or paralysis of the insects and the diarrhea that accompanies the disease. Upon dissection, the digestive tube of a stricken larva appears abnormally distended and is often completely free of contractile movements. It also is of a lighter green color, and the contents are slightly more acid than in normal silkworms. The hemolymph is more viscous, coagulates rapidly, and is considerably reduced in volume compared with that of normal larvae. The blood cells are noticeably altered, the protoplasm appearing vacuolated and occasionally filled with refringent inclusions. The general symptoms are similar to those described by Pasteur for the disease he called "flat death" or "white death." It does not, however, appear to be the same as a similar condition ("*flacherie typique*") which, according to Paillot, has been described by Acqua and which consists in a malfunctioning of the Malpighian tubes with a subsequent physiological poisoning of the insect.

As has already been stated, the true cause of the disease is not known. Such factors as food, rearing methods, ventilation, and excessive temperature and humidity have been investigated, but none proved to be instrumental in bringing on the disease. Paillot believes that some factor similar to asphyxiation might be concerned since the development of the disease in a colony progresses so rapidly. No microorganisms seem to be involved, and the disease cannot be transmitted by the transfer of blood from a diseased to a nondiseased insect. The ingestion of diarrheic intestinal contents does not give rise to the disease.

Histological sections of the alimentary tract of a diseased individual show a marked destruction of the calciform cells of the intestinal epithelium to have taken place. In the anterior portion of the midgut the mitochondria may appear in the form of rounded grains of variable size; the filamentous

mitochondria become fragmented. In the central portion of the midgut the mitochondria are reduced to small, variable-sized, rounded grains or are grouped together into rounded vesicles that represent marked mitochondrial degeneration. In this portion of the midgut a few fuchsinophilic blocks, such as those which occur in flaccidiform dysentery, may be seen. Occasionally the striated border shows a few fuchsinophilic blocks present. Other cellular changes occur in the intestinal epithelium, among which are a well-vacuolated cytoplasm, and occasionally the nucleoli

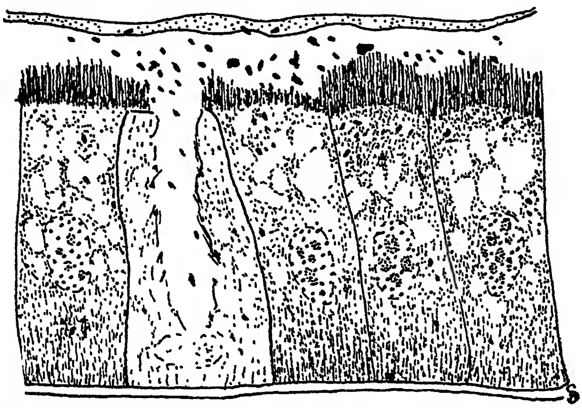

Fig. 26. Diagrammatic cross section of midgut of a silkworm suffering from pseudoflacherie. (Normal aspect may be seen in Fig. 24A.) Note pathological changes as described in text.

are rounded and hypertrophied in some cells. Most of the remaining nuclear characters are unchanged.

In the muscles of diseased larvae the mitochondria appear in the form of long threads of fuchsinophilic grains. According to Paillot, the destruction of the mitochondria explains the relaxation of the muscle fibers. Other minor cellular alterations may be noted in the muscle cells. The adipose tissue is generally more altered in the cephalothoracic region of the insect than in the abdominal part; while the chondriocontes of that in the abdominal region appear in the form of long flexible filaments, those of the anterior cells are largely transformed into small fuchsinophilic blocks. When the adipose cells have arrived at an advanced stage of alteration, mitochondria of regular size are often accumulated about the nucleus, the undifferentiated cytoplasm forms a thickened layer around the mass, and in the nucleus the chromatin granules appear smaller than normal. The silk glands are altered only very slightly, at least in the secretory portion. Here the mitochondria appear to be a little more broken up than in normal insects.

Valentian Dysentery. This ailment takes its name after the town of Valence in France where it was first observed in 1933 by Paillot (1942), who called it "*la dysenterie valentinoise.*" It appears to be a condition brought on by too high a temperature in the insectary at the time of molting. That such a situation may adversely affect a colony was noted early by Pasteur, who mentioned that the insects do not die immediately but become weakened so that they are later susceptible to flacherie. The disease may break out suddenly at the end of the fourth instar, and at first the death rate may be very high. The external symptoms are similar to those of pseudoflacherie in that larvae at the point of death are distinguishable from normal insects only by their almost complete immobility and their lack of response to stimuli. The diseased larvae differ from those afflicted with pseudoflacherie, however, in that the volume of blood remains about normal instead of being markedly reduced. The two diseases also differ in the types of histopathological lesions they produce in the intestinal epithelium.

The intestinal contents of the larvae generally contain abnormally large numbers of bacteria. This is one of the results, however, and not the cause of the malady or of the cellular lesions. *Streptococcus bombycis* is one of the bacteria most frequently seen in this situation.

Dysentery of Embryonic Origin. As in the case of the above-mentioned disease, in 1933 Paillot (1942) observed a condition, which he designated as "*dysenterie d'origine embryonnaire,*" in the vicinity of Valence, France. This malady in silkworm larvae apparently was concerned with their embryological development and, in fact, was traced back to a particular lot of eggs. Paillot believed that an abnormal acceleration of the embryogenic process during the incubation period is largely responsible.

The general symptoms of the malady are similar to those of the infectious dysenteries, but the histopathological lesions are characteristic and distinct. Rather intense cellular destruction occurs in the cells of the midintestine. The disease probably makes its appearance only on rare occasions and then to a limited degree.

Genetic Abnormalities. Abnormal genetic phenomena, as they occur naturally in insects, have not been very well studied. To be sure, numerous observations have been made on the alterations and variations that occur in insects as indicated by structural or color changes. Sometimes these abnormalities are extremely marked in degree and bizarre in form. Some modifications are merely accidents or injuries of birth, but occasionally the absence or malformation of an insect part may be due to malfunctioning genetic factors. In other cases such malformations as tumors have been shown to be hereditary in drosophila flies; the position, in the chromosome, of the gene for the tumor has even been determined (Stark, 1919).

It is our purpose here to mention briefly a malady of the honeybee, *Apis melifera* Linn., which Butler (1943) has designated as a genetic type of bee paralysis. First described in Marburg by Dreher, this malady may be noticed by the trembling of the body and wings of the bee and by the insect's sprawled appendages. The bees are found emerging from the cells in an almost hairless condition. The hair has not fallen off; rather it has

Fig. 27. A portion of *Hyalomma* sp., in dorsal aspect, showing milky-white elevations or papules. Inset shows a papule from the ventral integument as seen in section, showing it to consist of chitin. (*Drawn from illustrations by Olenev and Rozhdestvenskaja, 1933.*)

never been formed. The affected bees have a markedly reduced vitality, and drones as well as workers may be afflicted. The colonies contain numbers of shiny black bees and yield very little surplus honey. The cause is thought to be a hereditarily faulty queen. This form of paralysis apparently occurs only in rare instances. It may be rectified by requeening the colony with a young, healthy, unrelated queen.

Diseases of Unknown Etiology. There are, of course, many conditions in insects which might logically be placed under the heading of this paragraph. When, for example, we say that an ailment is due to a deranged metabolism, we may not know the exact cause of the ailment, but we do

know that it was brought about by a malfunctioning metabolism. Here, however, we refer to those conditions for which it has been impossible to ascertain whether the cause is microbial, chemical, physical, or due to a faulty metabolism. In some situations it has been impossible to know if one is working with an infectious or a noninfectious condition. Not many such situations have been reported in insects, but they probably exist. The following example might be considered to fall in this category.

In 1933 Olenev and Rozhdestvenskaja reported a peculiar pathological condition in several specimens of female ixodid ticks (3 *Hyalomma* sp.; 1 *Dermacentor niveus* Neum.). The integument of these ticks harbored numerous round to oblong milky-white elevations, which the authors called "papules." The papules occurred only on the thinly chitinized parts of the ticks' bodies and were not present on the capitula, scuta, or legs. The papules varied in size from very small ones visible only under a dissecting microscope to large ones easily visible to the naked eye. One tick had in the neighborhood of 1,000 papules on its body. Cross sections through a papule showed it to consist of chitin (Fig. 27). The cause and significance of these peculiar formations have not been ascertained.

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CHAPTER 4

THE EXTRACELLULAR MICROBIOTA OF HEALTHY INSECTS

It scarcely need be pointed out that unless one has a thorough understanding of the types of flora and fauna usually present in or on normal insects, one cannot hope to gain an accurate knowledge of the microbiota of abnormal or diseased insects. In the present volume no attempt is made to treat the normal microbiota in a manner that is in any sense complete. To some extent this has been done in other works to which the reader is referred (*e.g.*, Buchner, 1930; Paillot, 1933; and Steinhaus, 1940, 1946). It is, however, necessary at this point to familiarize the student of insect pathology with certain aspects of this phase of insect microbiology and thus to make more understandable many of the pathological aspects of the microbial diseases with which the greater share of this book is concerned. We shall attempt to accomplish this in the present chapter and in the next.

The word "microorganism" is usually used in referring to any organism of microscopic or ultramicroscopic size. Included are the bacteria, fungi (yeasts and molds), protozoa, spirochetes, rickettsiae, and in most cases the viruses. Some authors would include the nematodes, although these are metazoans whereas most of the microorganisms are unicellular. At any rate, in the microscopic world we have a population that in the number of known species comes as near to approaching that of insects as does any other category of living things. As concerns total numbers, the microorganisms far exceed even that of insects and they are also more ubiquitous than are the latter. It is not at all strange, therefore, that the two forms of life, insects and microorganisms, should be found in so many relationships with each other.

These relationships or associations are as varied as they are numerous. Some of them are of great importance to the participants while others are of only an adventitious or fortuitous nature. The association may be a direct or intimate one, or it may be quite indirect. Instances of the latter are seen in cases where microorganisms bring about a breakdown or decay of substances which may then become available as food for insects. In other cases the microorganisms are not attached to or located within the insect but may be located elsewhere as in the case of the fungous gardens of certain wood-boring beetles and ants. These gardens are cultivated in certain parts of the gallery and are carefully tended by the insect.

Those microorganisms which are found on or in the insect host itself may conveniently be divided into two large groups which we shall designate as (1) the extracellular microbiota and (2) the intracellular microbiota. This grouping is based on the characteristic location of the microorganisms, the extracellular occurring outside the tissue cells of the insect and intracellular occurring within the cytoplasm and sometimes the nucleus of the tissue cells of the insect. Occasionally no distinct line can be drawn between these two groups since some microorganisms occur both extracellularly and intracellularly and some may be found in an extracellular position at one time and in an intracellular position at another time in the cycle of its association with its host. The intracellular forms will be discussed in the next chapter; here we shall be concerned with the extracellular microbiota only.

The extracellular microbiota may be further divided into (1) the external microbiota (*i.e.*, situated on the exterior of the insect's body), and (2) the internal microbiota (*i.e.*, located on the interior of the insect's body).

EXTERNAL MICROBIOTA

Bacteria

The number of bacteria living on the external surfaces of insects in most cases is surprisingly small, although some insects, such as the housefly, may carry relatively large numbers. Even the filth-frequenting housefly, however, usually harbors more bacteria internally than externally. This is due largely to the more suitable conditions for microbial growth which exist in the insect's alimentary tract. In many instances the brushlike appendages of certain insects come in contact with and acquire large numbers of microorganisms as they move about in their environment. This is not always the case, however, since it is known that, even though the body structure of bees is well adapted for the carrying of pollen, comparatively few bacteria are found on their external surfaces.

No thorough study has been made of the external flora of insects in general, but there are indications that the gram-positive sporeforming group probably predominates in most insects. Of course, the environment of an insect largely determines the type of microbiota found on it. Soil-inhabiting insects usually harbor soil microorganisms. On insects living on animals one might expect to find the types of bacteria usually associated with the skin or fur of the animals. Such insects as houseflies and cockroaches, which frequent filth, will carry a flora more or less characteristic of that occurring in their surroundings. In "clean" areas they are likely to have a different flora from that found on the same species of insect living in an area of filth.

Fungi

Most of the fungi that have been studied in their association with insects are parasitic or semiparasitic on their hosts. In some of these cases the host may be said to be diseased. Normal insects, however, frequently harbor fungi externally. In fact, representatives of any of the four classes of fungi (Phycomycetes, Ascomycetes, Basidiomycetes, and Deuteromycetes) may be found in this location. Even the pathogenic entomogenous fungi may, after growing internally, break through the integument and appear to be located on the exterior of the insect. Relatively few fungi, however, germinate, grow, and reproduce entirely on the insect's external surfaces. Usually fungi found in this location are in the spore stage or in some other more or less quiescent state. Sometimes large numbers of fungous spores are carried by the insects, but usually the number is comparatively small. As in the case of the bacteria, the type of fungi found on the external surface of an insect depends largely upon the nature of its habitat. Perhaps the spores of the Fungi Imperfecti are those most commonly present on normal insects in nature.

It might be mentioned that numerous fungi live on the exuviae of dead insects. Indeed, bacteria and fungi undoubtedly play the predominant role in final disintegration of the bodies of dead insects turning the elements of these animals back to nature.

Laboulbeniales. One group of fungi that does grow and multiply almost entirely on the external surfaces of insects is that designated by the ordinal name Laboulbeniales. Although considered by some to be parasitic in nature, these exclusively entomophilic fungi may also be considered as commensals that are in most cases harmless to the host. They are true parasites in the sense that their existence depends upon the continued life of the host. When the latter dies, the fungi also succumb. Also, they may be considered as causing cutaneous diseases since they live largely on the chitinous cuticula of living insects and are transmitted from one individual to another. On rare occasions, with certain soft-bodied insects, they penetrate the interior with extensive rhizoidal processes of the basal cell and may ramify throughout the fatty tissue or gain nourishment from the blood of the insect. In most cases, however, the life of the fungus is relatively secure and long since it causes so little inconvenience to its host that its length of existence is that of the insect.

The first published note on these fungi was that by Rouget in 1850. This was followed 2 years later by Mayr's report on another species. Neither of these workers, however, realized the true nature of these organisms as did Robin in 1853. In this last worker's publication the new genus *Laboulbenia* Montagne & C. Robin was erected in honor of La-

boulbène, the entomologist, who was perhaps the first to observe *Laboulbenia rougettii* of these authors. This fungus occurs on a species of ground beetle (*Brachinus*) in Europe. In the years following 1853 a few other authors published accounts of these fungi, but it was not until the monumental work of Thaxter appeared that a real knowledge of the group was obtained. His monograph of the Laboulbeniaceae appeared in five parts in the years 1896, 1908, 1924, 1926, and 1931. Most of our present-

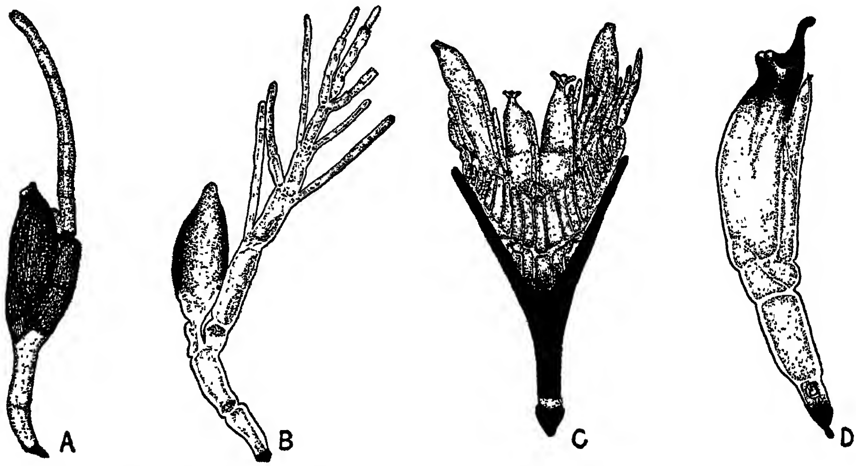


Fig. 28. Examples of laboulbeniaceous fungi. *A. Laboulbenia vulgaris* Peyritsch on carabid beetles. *B. Cantharomyces permasculus* Thaxter on *Parygrus*. *C. Dichomyces hybridus* Thaxter on *Philanthus*. *D. Chitonomyces atricornis* Thaxter on *Orectogyrus*. (*Redrawn from Thaxter, 1896, 1908, 1926, and 1931.*)

day information stems from Thaxter's work. In 1934 Colla also presented a detailed account of the group.

The laboulbeniaceous fungi are small, frequently minute, organisms belonging to the class Ascomycetes. On the insect, they appear as scattered or as densely crowded bristles or bushy hairs which, on certain areas of the host's integument, form a furry or velvety patch. Their site of attachment is usually limited to definite regions on the integument of each host species; *i.e.*, the distance of the fungus from the margins of the elytra, for example, is always about the same, and a species that is usually found living on the left elytron will rarely be found living on the right elytron.

The main body, or receptacle, of the fungus is fixed to the integument of the insect by means of a blackened base, or foot, and in most cases consists of a very small number of cells, differently arranged in different genera. This receptacle gives rise to appendages of variable sizes and shapes, which support the male and female reproductive organs. The male and female organs occur on the same individual, except in a few cases in which the

plants are dioecious. The perithecia eventually develop from the female structures and may arise singly or in considerable numbers from a given individual. Within the perithecia the reproductive bodies, or ascospores, are formed in asci identical in most respects to the asci of other Ascomycetes.

Transmission of the fungi from one insect to another occurs through the agency of the spores, which at times of direct contact between insects, such as during copulation, are discharged or forced out of the asci. The spores are fairly uniform in size and shape. They are hyaline and are fusiform or acicular in form. Usually they are divided into two cells of unequal size by a septum or pseudoseptum. The contents of the spore generally consist of a fairly homogeneous granular protoplasm. The spore itself is nearly always surrounded by a gelatinous envelope characteristically thickened about its base. This envelope serves as a protective covering and also assists the spore in adhering to the new host insect. Germination of the spore commences with a modification of its lower extremity into an organ of attachment known as the "foot." Because of the change that takes place in this end of the gelatinous envelope, the base becomes blackened, opaque, and hardened as it attaches the growing plant firmly to the integument of the insect.

Each species of fungus appears to be limited to a certain genus of insects. Most of the hosts are Coleoptera and Diptera.

The order Laboulbeniales has been divided into three families on the basis of the relative types of development of the male sexual apparatus: Ceratomycetaceae, Peyritschiellaceae, and Laboulbeniaceae. The Ceratomycetaceae are characterized by the fact that their antherids (male sex organs) are more or less undifferentiated cells of the appendages or their branches. The antheridial cells of the Peyritschiellaceae are endogenous and are united in a specialized organ. Because they extrude their spermatia into a common chamber before liberation they are known as compound antheridia. Some members of this family, especially those occurring in the tropics, are fairly large.

Laboulbeniaceae is the largest and best known family. Its members are characterized by having antherids that are differentiated single cells with free efferent tubes. Members of this family have been found on insects of the orders Coleoptera, Diptera, Neuroptera, Orthoptera, Isoptera, and Hymenoptera, as well as on certain Arachnida. The well-known genus *Laboulbenia* is commonly found on Carabidae or ground beetles as well as on numerous other insects.

The distribution of Laboulbeniales apparently is world-wide. Frequently the distribution of these fungi corresponds to the distribution of the genera of insect hosts. For example, *Laboulbenia cristata* Th. occurs in

all continents on the large and widespread insect genus *Paederus*. *Laboulbenia pheropsophi* Th. may be found almost wherever its *Pheropsophus* hosts occur in five continents. Others, such as *Laboulbenia variabilis* Th., occur on a variety of hosts but are not found outside the American continent.

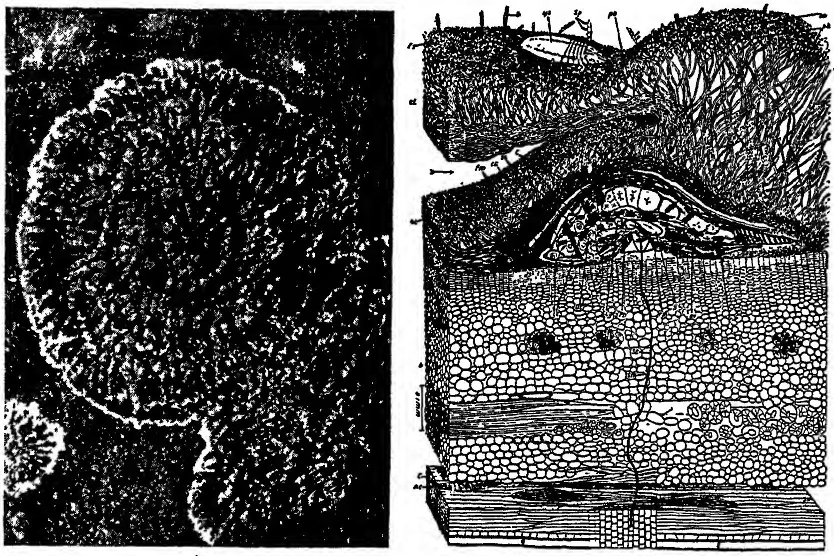
Septobasidium. The genus *Septobasidium* belongs to the family Auriculariaceae and to the order Tremellales of the class Basidiomycetes. It is perhaps the most noteworthy group of Basidiomycetes to be associated with insects, in this case with scale insects that live beneath the stromata of the fungi. This association was first reported by Von Höhnel and Litschauer in 1907, and soon thereafter their observations were confirmed by other workers. In 1929 and 1931, Couch studied in detail the relationship between *Septobasidium burtii* Ll. and the scale insect *Aspidiotus osborni* New. & Ckll. Then, in 1938, Couch published a treatise on the genus *Septobasidium* which presented a comparative study of the species of this genus as well as a careful treatment of the biological relationships involved. It is from this work that most of our information has been obtained.

The different species of *Septobasidium* vary considerably in size; some are very small (3 to 30 millimeters), while others are small but, because of the large anastomosing patches formed, are quite conspicuous. Still others form individual patches of 20 centimeters or larger, some accumulating to cover extensive areas of the host tree. Some species have a characteristic and definite shape while the outline of growth of others is very indefinite and irregular. The thickness of growth of the fungi also varies with the species. About half the known species show three distinct structural regions or layers. Some species are brilliantly colored: golden yellow, reddish-purple, grayish-blue, and tawny. Some are almost black and some are nearly pure white when fresh. The most common color is some shade of brown.

In the United States, one of the best studied species is the above-mentioned *Septobasidium burtii* Ll. This fungus is a perennial, as are probably most species, and its period of growth in southeastern United States is between April and November. Its most luxuriant growth is usually attained on the undersurface of the lower branches of trees, rarely occurring on the main trunk. Normally it reproduces by the formation of spores that develop after rains and while the fungus is damp. The body of the fungus consists of oblong or circular resupinate patches measuring up to several centimeters in diameter. Each patch is made up of varying numbers of irregular concentric rings of growth, a new ring being formed each year (Fig. 29*A*). The fungus is composed of top and bottom layers between which are numerous tunnels and chambers, many of which are in direct communication with the outside. These chambers contain the scale insects (in this case *Aspidiotus osborni* New. & Ckll., and rarely *Chry-*

somphalus obscurus (Comst.)), usually one, but sometimes two or three, to a chamber. The shape of each chamber is adapted to the shape of the insect's body but is somewhat larger.

The relationship between the fungus and the scale insects has been assumed by many to be one of parasitism. Couch (1931), however,



A B

Fig. 29. *Septobasidium burtii* Lloyd growing on the bark of a tree (*Quercus*). *A*. Surface view of fungus, showing radiating ridges and openings to tunnels. If the top layer were removed, numerous scale insects would be exposed. *B*. A diagrammatic sectional view showing the association between the fungus and the scale insect, *Aspidiotus osborni* N. & C. The sucking tube of the insect may be seen extending down through the bark of the tree into the cambium region. A young scale insect may be seen crawling over the fruiting surface of the fungus. (*From Couch, "The Genus Septobasidium," 1938, University of North Carolina Press, Chapel Hill.*)

maintains that the fungus and the insects live in a state of mutual symbiotism at the expense of the host plant. In return for the furnishing of a protective home, the fungus receives from the insects a source of food and a means of distribution. In obtaining its food the fungus parasitizes a considerable number of scale insects. This sacrifice is for the good of the colony as a whole, since it enables the fungus to grow and to form more houses for the nonparasitized as well as the parasitized insects. Unless the insects are infected when young they apparently remain free of the fungus. Thus the fungus and the insects live together interdependently.

As shown in Fig. 29*B*, the parasitized insects may be firmly embedded in the fungus with their sucking tubes extended into the bark of the tree.

Hyphae of the fungus enter the circulatory system of living insects through the dermal pores (other routes of entry have been observed with other species) and there develop numerous coiled haustoria that are connected end to end by very delicate hyphae or sometimes gathered in clusters of two or three. Because the growth of the fungus within the parasitized

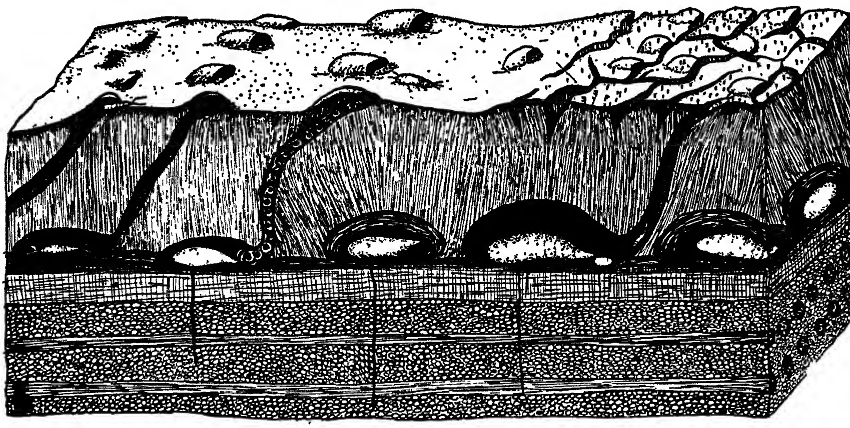


Fig. 30. Diagrammatic representation of *Septobasidium fraxini* Couch showing certain of the relationships between it and the associated scale insect. As described by Couch (1946): "Top surface (left) shows four crescent-shaped entrances to insect houses, with section view of a fifth and part of a tunnel with young insect entering. Next are shown three older entrances which have become igloo-like in shape; a fourth is shown in section with a young insect that has just settled in the chamber below. In center above are two entrances, the top one partly blocked by white, waxy coils, the second with a young insect headed for it, and a section of a third with a tunnel leading down to the chamber occupied by an insect; entrance and tunnel partly blocked by white coils. Insect in center below completely covered by fungus and parasitized. To right, fruiting surface of fungus, showing basidia and basidiospores. Below is a section of a large fungal house containing an adult female giving birth to young, two of which are making their exit through tunnel. Black threads extending into bark from insects are sucking tubes of insects." (*Redrawn from Couch, 1946.*)

insects is very slow, such insects continue to live throughout the dormant season sucking the juices of the host plant. The bodies of markedly parasitized insects are dwarfed and incapable of reproduction. The nonparasitized insects are apparently entirely free of fungus internally and are nowhere in contact with the fungus that covers their bodies.

In order to become infected, the young scale insects must come in contact with the bud cells formed by the fungous spores. Apparently they are never infected directly by the hyphae. The young insects may come in contact with the bud cells when they crawl out through the opening of the tunnel over the surface of the sporebearing fungus. According to Couch (1938), these infected insects may crawl back beneath the fungus

colony under which they were born, or they may settle down beneath other fungus colonies, or they may situate themselves on the bark where there is no fungous growth. In the first two instances the insects are responsible for the survival and continued growth of the fungus colonies already established. The third group settles down on the bark and starts a new colony, thus affecting the distribution of the fungus. Since the dissemination of the wingless young is limited, transportation of *Septobasidium* for great distances probably depends upon the transplanting of infected plants.

The damage caused to the trees on which *Septobasidium* and the insects live and grow may be severe, or it may be light and inconsequential.

Approximately 300 species of *Septobasidium* are known in various countries of the world. About 40 of these species are found in the United States, so far mainly in the southeastern part. The most common species in this country appears to be *Septobasidium curtisii* (B. & D.). Other common ones are *S. pseudopedicellatum* Bur., *S. sinuosum* Cou., *S. apiculatum* Cou., *S. castaneum* Bur., and *S. alni* Tor. Most of these live on several different species of trees, although some, such as *S. canescens* Bur., *S. grandisporum* Cou., and *S. sabalis* Cou. live on only one. Couch (1938) has found 76 species of trees to be subject to attack by species of *Septobasidium.*

About 20 species of scale insects have been found associated with *Septobasidium* in the United States. For the most part these are included in the genera *Aspidiotus, Cerecoccus, Chermes, Chinoaspis, Chrysomphalus,* and *Lepidosaphes. Aspidiotus* contains most of them, *e.g., A. anacylus* (Putn.), *A. juglans-regiae* Comst., *A. osborni* New. & Ckll., *A. forbesi* Johns., and others. Perhaps most species of *Septobasidium* are associated with several species of scale insects, but some species are associated with only one insect species. Thus *Septobasidium alni* Tor. and its variety *squamosum* Couch are found with at least seven different species of scale insects. As many as three different species of scale insects have been found under the same specimen of *Septobasidium apiculatum* Couch.

Ambrosia Fungi. Certain fungi should be mentioned here since, even though they do not live on the external surfaces of insects, they are associated with and tended by the insects externally. We refer to those fungi which are grown and cultivated by certain insects (particularly certain beetles, ants, and termites) to be used by them for food or other purposes. First we shall consider this relationship in which beetles are concerned.

In 1836 Schmidberger observed the larva of a beetle to feed upon a peculiar glistening white substance which, not knowing its true nature, he called "ambrosia." This "food of the gods" was thought by Ratzeburg (1839) to be the result of a mixture of insect spittle and plant sap. Then, in 1844, Hartig showed this material to be of a fungous nature, and he gave

the name *Monilia candida* to the fungus associated with *Xyleborus dispar.* It remained, however, for Hubbard (1897), Neger (1908–1911), and Schneider-Orelli (1911, 1913) to clarify more fully the interesting biological relationships existing between the ambrosia beetles and the ambrosia fungi.

The true ambrosia beetles, sometimes called "timber beetles" because

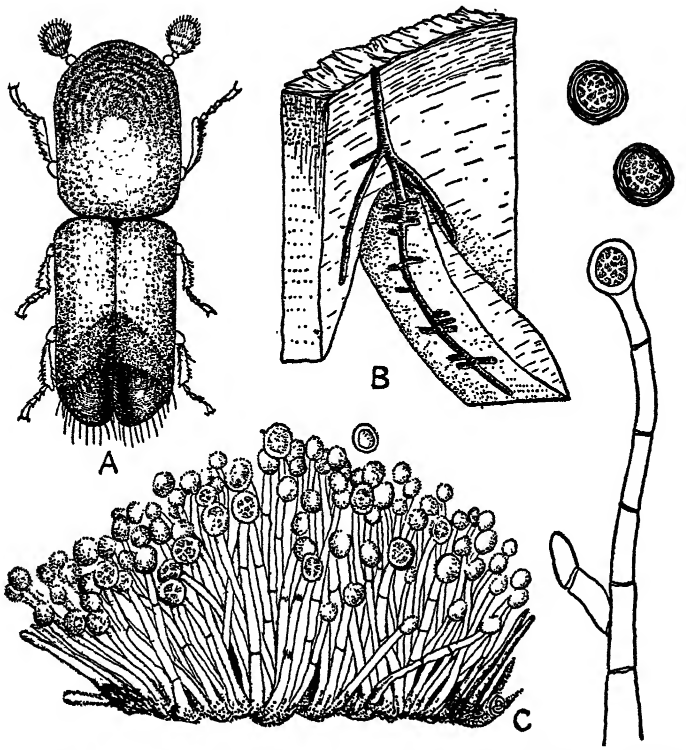


Fig. 31. Ambrosia beetles and fungi. *A*. An adult ambrosia beetle, *Monarthrum fasciatum* Say. *B*. Gallery of same beetle in maple. *C*. Ambrosia fungus of *Xyleborus xylographus* Say; greatly enlarged. (*Redrawn from Hubbard, 1897.*)

they burrow in the solid wood of trees, belong to the family Scolytidae (Ipidae), although some are also in the family Platypodidae. They usually attack weakened, sickly, or sometimes dying trees, burrowing deep into the sapwood and forming galleries characteristic of each species. In most instances there is a main gallery which may be branched and which extends deep into the solid wood. Numerous short galleries or chambers, called "cradles," extend from its sides. In each cradle an egg is laid and a larva undergoes its development. During this time it is constantly attended by the mother beetle, which plugs the mouth of each cradle with a mass of the ambrosia fungus. This plug of fungus is used as food by

the larvae, which from time to time perforate it to clear their cells of excrement pellets and other refuse. This refuse is cleaned away by the mother beetle, which constantly renews the supply of fresh fungus.

Some ambrosia beetles rear their young in large communal galleries where the young and old insects live together. When a member of the colony dies, it is sealed off in a special death chamber. The larvae reared in these communal chambers apparently depend solely on the fungus for all the nutrient elements. In the case of the larvae which live in separate chambers and which eat wood, the fungi serve as a source of nitrogenous material supplementing the wood.

The galleries are usually bored by the female beetles, although in some species the males may help. The adults enter the bark of the tree, leaving small "shot-hole" entrances similar to those made by bark beetles. Under favorable conditions excavation of the tunnels may proceed during two or three generations. The galleries of the ambrosia beetles may be differentiated from those of other wood-boring insects by the uniform size of the various ramifications and by the absence of refuse material and wood dust. In addition, their walls are always stained a dark color, usually brown or black, by the ambrosia fungus. The ambrosia fungi themselves do not invade the wood more than a short distance from the galleries. Although the gallery walls are deeply stained, they apparently are uninjured by the activities of the fungus, which lives on the contents of the sapwood cells but does not destroy the cell walls.

Among the most common ambrosia beetles in the United States are those in the genera *Xyleborus, Platypus, Corthylus, Monarthrum, Trypodendron,* and *Grathotrichus.*

Most of the ambrosia beetles cultivate the fungi in carefully prepared beds or gardens. Each species of beetle cultivates only one species of fungus, and only the most closely allied species of beetles cultivate the same fungous species. Somehow all extraneous or contaminating fungi are suppressed, although such secondary fungi overgrow the ambrosia fungi as soon as the galleries are deserted by the beetles. The fungus garden is started by the mother after she carefully makes a bed or layer of chips upon which she then deposits some conidia. New beds are started using the excrement of the larvae as a substratum. There are a variety of opinions as to just how the mother inoculates the bed or substratum. Some believe that the mother does this by regurgitating the spores from their temporary storage place in the crop. Others think the spores are distributed from the body through the fecal pellets. Still others believe that at least some species carry the spores and mycelium of the fungi on chitinous bristles on the front part of the head, and it is supposed that these are used to seed the new beds.

For some reason the ambrosia fungi have had relatively little attention from systematic mycologists. As a result, there is a marked dearth of information on them from a taxonomic viewpoint. A few scattered reports indicate that some of them, at least, may be of the genus *Monilia* or of other categories of the Fungi Imperfecti.

Hubbard (1897) distinguished two principal types among the ambrosia fungi: "(1) Those with erect stems, having at the termination of the stems, or their branches, swollen cells (conidia). (2) Those which form tangled chains of cells, resembling the piled-up beads of a broken necklace." Those beetles whose larvae are reared free in communal galleries (*Platypus* and *Xyleborus*), are associated with the erect type. Those whose larvae live in separate cradles (*Corthylus*, *Monarthrum*, etc.) are found among the beadlike type.

The growing parts of the fungus are juicy and very tender. Young larvae nip off the tender conidial tips while the older larvae and adult beetles eat the entire fungus down to its base from which it rapidly grows up again. The growth of the ambrosia fungus has been likened by Hubbard to that of asparagus, which remains tender and edible only when continually cropped but is no longer desirable as a food when permitted to go to seed. Similarly, if the ambrosia is allowed to ripen, it can no longer serve as food for the insects; in fact, it may be a source of danger to them by choking off the galleries and suffocating the inhabitants.

Ants and Fungi. Fungus-cultivating habits similar in some respects to those we have described for the ambrosia beetles occur among certain American ants living mostly in the tropics but in some cases extending up into the United States. These fungus-growing and fungus-feeding ants belong to the tribe Attini of the subfamily Myrmicinae. Over 100 species of attine ants have been described, and all are fungivorous. A different fungus appears to be maintained by each species or group of closely related species.

These ants, particularly those of the genus *Atta*, build large nests deep in the ground where they excavate cavities which may be as large as a good-sized water pail. The ants cut pieces from the leaves of trees or other foliage and carry them like parasols to their nests. For this reason these ants are sometimes called the "leaf-cutting" or the "parasol" ants. In the cavities of their nests they cut the pieces of leaves into smaller fragments from which they construct brownish spongelike masses which form the substratum of their fungus gardens. These spongelike masses are "seeded" by the female and are soon covered with a white mycelium. The workers carefully and continuously tend this growth, weeding it so as to keep out or suppress all contaminating growths, and treating it so that the hyphae produce large numbers of small spherical dwellings known

as "bromatia" (Fig. 32*B*, *C*). These bromatia, or as popularly called by some, "kohlrabi globules," are used as food by the ants and are fed to the larvae. That the bromatia are probably induced by the ants is indicated

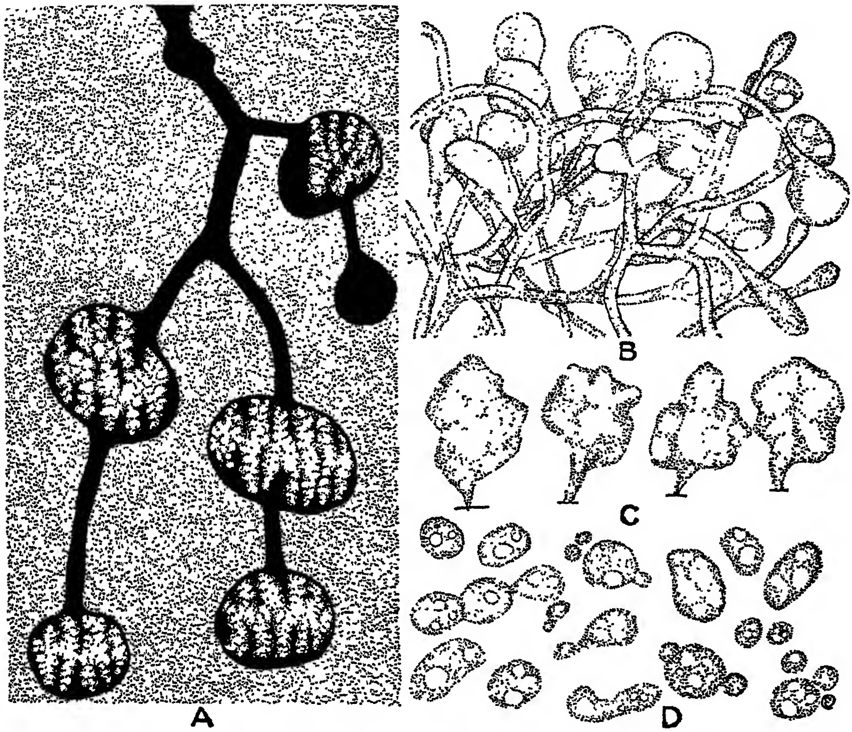


Fig. 32. Fungi cultivated by fungus-growing ants. *A*. Diagram of a large nest of an ant (*Trachmyrmex*) in southern United States, showing five chambers with pendent fungus gardens and a newly excavated chamber in which the garden has not yet been started. *B*. Modified mycelium (bromatium) of the fungus cultivated by an Argentinian ant, *Moellerius* (according to Bruch, 1922). *C*. External appearance of the bromatia of one of the fungi (*Tyridiomyces*) cultivated by *Cyphomyrmex* ants on insect excrement. *D*. Yeastlike cells that make up the bromatia shown in *C*. (*A*, *C*, *and D*, *redrawn and adapted from Wheeler, 1926; courtesy of the Columbia University Press.*)

by the fact that they are not formed when the fungus is grown on artificial media.

The fungus is transferred by the virgin queen at the time of swarming. When she is about to leave the parental nest, she takes a mass of hyphae and leaf tissue into her mouth and retains it in her infrabuccal pouch, which is a cavity, or spherical sac, below the floor of the mouth but opening into the mouth. Most of the time it acts as a repository for particles of solid material that are not ingested by the adult ant. After mating and

discarding her wings, she makes a small chamber for herself in the soil. Here she uses the saved pellet as spawn to start a new fungus garden. As the garden begins to grow, she fertilizes it with her feces or occasionally even by breaking up an egg and adding it to the garden. The garden rapidly develops, and soon it is large enough to serve as a nest for the first-generation eggs. The larvae hatching from the eggs eat the fungus, pupate, and develop into small workers, who go out and bring in more pieces of leaves to add to the garden. The queen henceforth devotes her time to laying eggs, and the workers assume the duties connected with the fungus garden.

Although more than 100 fungus-growing ants have been described, in only a few instances have the biological relationships between the ants and the fungi been thoroughly studied. For a list of the species of ants concerned and for a further discussion of the subject, the reader is referred to the works of Wheeler (1907, 1923, 1937) and also to those of Weber (1937, 1938).

Concerning the fungi themselves, very little information of a systematic nature has been forthcoming. Some of them are of the nature of true mushrooms which have been observed fruiting on the ground above deserted *Atta* nests. In other instances the smaller fungi, such as the Fungi Imperfecti, appear to be concerned.

Termites and Fungi. On the basis of their food habits, termites may be separated into two large groups. One group feeds largely on wood and the associated fungi; the other feeds principally on fungi cultivated in special "gardens," which they tend with great care. Termites of the latter group usually live in large nests called "termitaria," which they may build above or below the ground in large mud-covered structures. In the termitarium are special compartments in which the fungi are cultivated on the excreta of the termites. This fecal substratum is inoculated automatically when the spores of the fungus are ingested by the workers and pass uninjured through the insects' intestinal tracts. The spores germinate, giving rise to a thick growth of fungus, which is then used as food for the royal, or first, reproductive castes and the young. The workers and soldiers feed on other plant material and do not use the cultivated fungus.

In the United States, most of the termites are of the wood-eating type. Wood penetrated by these termites frequently shows indications of rot produced by fungi, the spores and hyphae of which are carried on the bodies of the insects into their burrows. Patches of fungi subsequently arise, and hyphae from these invade the walls of the galleries. Mycological examinations of the exteriors, as well as the guts, of termites have yielded several species of fungi. Very little is known, taxonomically, of any of the fungi associated with termites.

Yeasts. The external flora of yeasts on insects has been very inadequately studied, but indications are that the number of species present in this location is not great. Wild yeasts may occasionally be found on the exterior of insects, but usually the association is a fortuitous one. In some cases insects (bees, wasps, ants, mosquitoes, and gnats) have been known to distribute or to carry in nature such yeasts as *Saccharomyces cerevisiae* Han. and *Saccharomyces ellipsoideus* Han. Drosophila flies may carry yeasts to grapes in vineyards. These yeasts cause a fermentation of the grape which provides optimum conditions for the developing larvae of the flies. Sometimes yeasts pathogenic for plants are found being distributed mechanically by insects.

Protozoa and Viruses

Very little study of any kind has been made of the protozoa to be found on the external surfaces of insects. A few reports, such as that of the suctorian, *Rhynchophrya palpans* Col., attached to the water beetle *Hydrophilus piceus* Linn., have been made, but they are few and far between. This protozoan may occur on the beetle in the presence of other Suctoria (*Periacineata linquifera* and *Discophrya ferrum-equinum*) as well as with certain ciliates (*Vorticella*). Undoubtedly other undescribed species of entomophilic Suctoria exist. Hydrophilid beetles are found also with certain species of *Epistylis* clinging externally to various parts of their bodies. Spores and cysts of certain freeliving protozoa may occur on the outer covering of insects in nature. Intestinal protozoa of man and other animals are probably acquired, at least temporarily, on the appendages of insects frequenting filth. Similarly, the mouthparts of bloodsucking insects may temporarily be contaminated exteriorly with protozoa from the blood stream of infected animals.

Possibly the only viruses that are to be found on the exterior surfaces of insects are those which arrive there by accident or fortuitous circumstances. Such might occur in the case of those viruses causing diseases of plants and animals, in which case the mouthparts or appendages of insects may become temporarily contaminated.

INTERNAL MICROBIOTA

The internal extracellular microbiota of healthy insects is usually greater and more varied than is the external microbiota. The greater part of the internal microbiota of insects is located in the intestinal tract. Almost any tissue, however, including the blood, may normally and regularly harbor microorganisms, although frequently they consist of what might be designated as intracellular microorganisms, or symbiotes. Extracellular microorganisms are found internally principally in the alimentary tract.

Bacteria

Although more than 200 species of bacteria have been described from normal healthy insects, there is considerable confusion as to the true identity of a large number of them. Moreover, the nomenclature is badly confused and often misleading. For instance, many species that do not produce spores have been placed by their discoverers in the spore-forming genus *Bacillus*. Many others have been placed in the genus *Bacterium* without much, if any, consideration as to the true generic status of the organism. Then too, since it often seems to be much easier to name a new species of bacterium rather than to identify an old one correctly, many names exist that are merely synonyms for recognized species. To a considerable extent, therefore, it would be meaningless to say that almost 100 species of the genus *Bacillus* and practically 50 species of the genus *Bacterium* have been isolated from healthy insects, since such a statement would not take into account all the taxonomic and nomenclatorial vagaries that encumber an accurate analysis of the groups concerned and which still remain to be clarified. Fortunately the great majority of bacteria that occur extracellularly in insects are readily cultivable on artificial media. Accordingly, they may be studied in pure culture with relative ease; and when more insect microbiologists and insect pathologists awaken to the need of the use of adequate methods of bacterial systematics, the situation will no doubt improve. In the meantime, it is apropos of our subject to consider briefly some of the relationships existing between healthy insects and their bacterial floras.

Bacterial Flora of Normal Alimentary Tract. The variations in structure and function of the alimentary tracts of normal insects undoubtedly have a great deal to do with the types of bacterial flora present in them. In some insects the tract is merely a tube extending from the mouth to the anal opening. In such insects the bacterial flora is usually of a very simple type consisting principally of common adventitious and saprophytic forms. In other insects the alimentary tract may be considerably more complex, with various kinds of pouches, sacs, caeca, and diverticula, and with numerous crooks and turns giving the tract a length much longer than that of the insect's body. In these insects one is likely to find a greater variety of bacteria, and sometimes the bacteria in the pouches and caeca are of a very peculiar and characteristic type.

The alimentary tract of most insects may be considered as having three main parts: foregut, midgut, and hindgut (or foreintestine, midintestine, and hindintestine). Since the foregut and hindgut are invaginations of the body wall, they have a chitinous lining that is continuous with the cuticula of the body wall. The midgut develops from an endodermal

tube, the mesenteron, and is usually lined with a layer of large epithelial cells bounded externally by a basement membrane. Toward the lumen, these cells may have a striated border, and protecting the lining from the food particles in the gut may be a thin membranous structure known as the "peritrophic membrane." The bacterial flora of the various parts of the tract may vary qualitatively as well as quantitatively; *i.e.*, an insect may harbor a species of bacterium in one part of the digestive tract different from that in another part of the tract. Furthermore, a bacterium may be present in large numbers in, for example, the hindgut but present in only small numbers in the midgut; or vice versa.

Considering the alimentary tract as a whole, it may be noted that similar qualitative and quantitative variations occur between the different species of insects as well as between different individuals of the same species. The gut of some insects is sterile. This is frequently the case with insects that suck blood or sap as their food. Certain biting and chewing insects may also have digestive tubes devoid of bacteria. Sometimes this sterility is limited to certain portions of the gut. Thus, in the case of blowfly maggots (*Lucilia*), at one time used in the treatment of osteomyelitis, the bacteria taken in with the food are destroyed while passing through the long tubular stomach of the maggot so that none survive as far as the hindgut. The active principle in this case was found to be a substance called "allantoin."

Certain insects and ticks produce in their alimentary tracts a peculiar bactericidal principle that kills, even in vitro, such bacteria as *Micrococcus pyogenes* var. *aureus* (= *Staphylococcus aureus*). When, for example, this bacterium is ingested by the fowl tick, *Argas persicus* Oken., it is soon killed within the arthropod's gut. In the stable fly, *Stomoxys calcitrans* (Linn.), and certain other insects, not only do the gut contents contain the bactericidal principle, but the feces do as well. The principle is not inactivated by exposure to a temperature of 58°C. for 30 minutes.

Although no large accurate qualitative analysis of the bacterial flora of the alimentary tracts of insects has been made, there does seem to be emerging some sort of general picture as to the different kinds of bacteria present in many insects. Perhaps the most generally distributed group in insects is that composed of the gram-negative small rods. In this respect the bacterial flora is similar to that of higher animals. Of this group, the coliform bacteria (*i.e.*, those which are similar to the common colon bacillus of man and other animals and which usually ferment lactose) and closely related organisms are the ones most frequently encountered. Next frequently seen are the micrococci and the sporeforming bacilli. Most of these are common saprophytic forms that occur ubiquitously in nature. Spirilli are found only rarely in the guts of normal insects.

Like most groups of bacteria, those associated with insects may undergo considerable variation in shape, size, and structure. Some entomophytic bacteria undergo marked morphological changes when introduced into a host other than its normal one. These changes may consist merely of an increase or decrease in length or over-all size; on the other hand, peculiar bizarre-shaped forms may result. With some bacteria involution forms appear when grown on artificial media. In a later chapter we shall have occasion to discuss important instances of variation that occur in certain of the bacteria that are pathogenic for their insect hosts.

It should be remembered that the type of bacterial flora within an insect is determined largely by its environment just as in the case of the external flora. Insects living in the soil are likely to harbor bacteria found in the soil; in those living in water one might expect to find water and soil bacteria; those living on animals could probably acquire a flora characteristic of the skin or fur of their hosts. In the case of filth-inhabiting insects, one is almost certain to find bacteria common to the filth itself. Thus houseflies and cockroaches are notorious for the variety of bacteria they contain. Houseflies usually possess a flora consisting principally of coccal forms during the early spring, but frequently this changes to one consisting largely of gram-negative small rods, mostly coliforms, by midsummer and late fall. Of course, the feeding habits of an insect are directly linked to its environment as concerns its bacterial population. Insects that are more fastidious in their food selection may have a flora less diversified than that of such scavengers as cockroaches and houseflies. Quite a variety of bacteria may be picked up by those insects which eat foliage, especially when compared with those insects which only suck the sap. The latter, like those insects which feed on blood, usually harbor very few bacteria and these are frequently all of one species. Since the feeding habits of insects frequently vary with the stage of the insect, it is not surprising to find the flora of a butterfly, for example, differing radically from that of the caterpillar that preceded it. However, many times the flora of the larva is retained by the pupa and carried through to the adult stage.

Properly considered as part of the bacterial flora of the alimentary tracts of some insects are those bacteria which live in peculiar saclike appendages known as "gastric caeca." These caeca are especially characteristic of the higher Hemiptera and are almost always filled with large numbers of bacteria. The bacteria are morphologically characteristic for the species of insect harboring them, and those from different hosts range from very small rods to large sinuous spirochetelike forms. They pass from generation to generation in association with the egg. Only a few forms (such as those from *Anasa tristis* (DeG.), *Chelinidea tabulata* (Burm.), and *C. vittiger* Uehler) have been cultivated on artificial media.

Those from some species have defied cultivation on any of the many kinds of media tried. The function of the caecal bacteria in relation to their hosts is not clear. That they play a nutritional role is suspected. Most of the work on this interesting group of bacteria and the caeca they inhabit has been of a morphological nature.

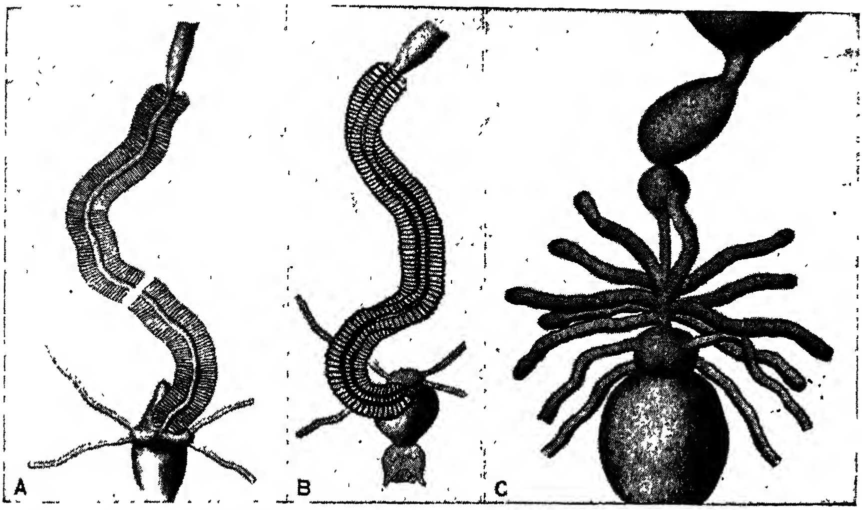


Fig. 33. Parts of the alimentary tracts of three species of Hemiptera showing three types of gastric caeca. *A. Anasa tristis* (DeG.). *B. Peribalus limbolarius* Stål. *C. Blissus leucopterus* (Say). (*From Glasgow, 1914.*)

Effect of Metamorphosis on Flora. The bacteria that are acquired by the immature stages of an insect may be destroyed or eliminated by an insect by the time it becomes an adult, or they may be retained all through the metamorphosis of an insect. (Only rarely, however, are the bacteria associated with the gut of an insect passed through the egg stage.) In the case of flies and certain midges, for instance, it has been shown that bacteria ingested during the larval stage may survive through metamorphosis into the adult stage and remain with the insect until its death. Not all species of bacteria are necessarily retained; certain types seem to be held by the insect more readily than do others. Undoubtedly a similar relationship exists between other species of insects and other bacteria. Only 15 or 20 instances of this kind have been reported. Sometimes this phenomenon is of considerable economic or public-health importance. An example of the former is the retention of *Erwinia carotovora* (Jones), the cause of potato blackleg, within the digestive tract of the overwintering puparia. An instance of the latter is the retention of bacteria pathogenic to man in the housefly from maggot to adult.

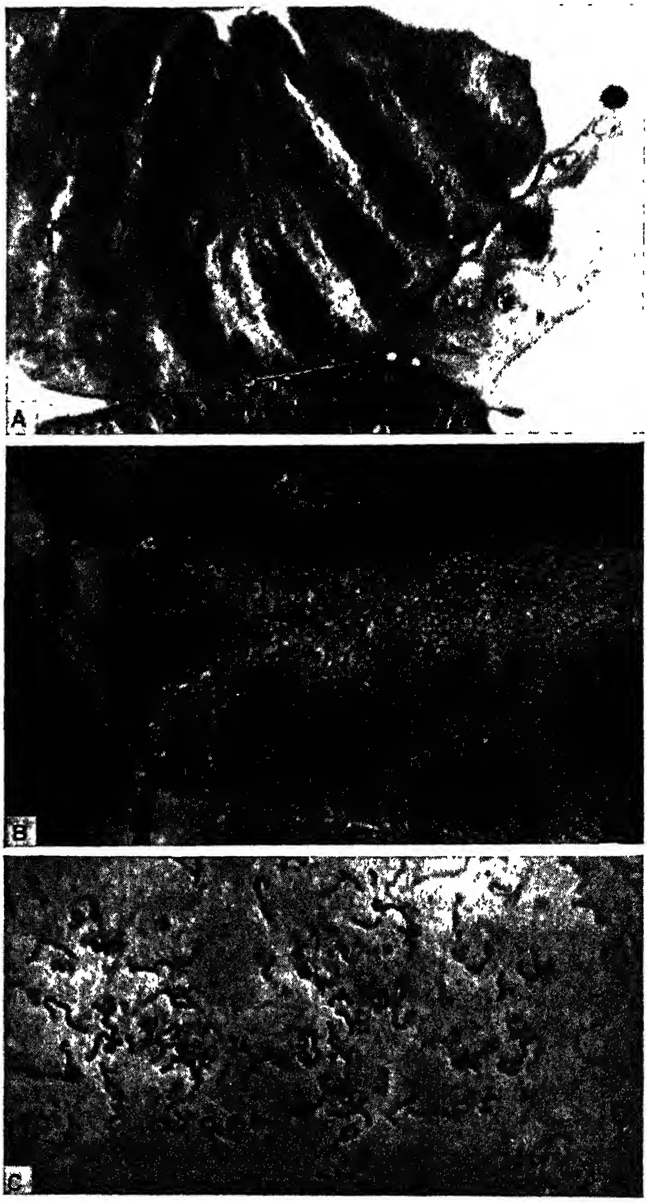


Fig. 34. Caecal bacteria of the harlequin bug, *Murgantia histrionica* (Hahn.). *A*. Stained histological section through the gastric caeca showing the separate compartments. *B*. Enlargement of one of the compartments showing how densely it is packed with bacteria. *C*. Bacteria from caeca of the harlequin bug as shown in stained smear. (*Photographs by K. M. Hughes and J. M. Smith.*)

Generation-to-generation Transmission. The transmission of intracellular microorganisms from one generation to the next is a common occurrence; it is much less so in the case of the extracellular bacteria associated with insects. In most instances of the latter the bacteria are carried over in the egg; sometimes the bacteria are located on the exterior of the egg, and sometimes the eggs are merely laid in the midst of an environment filled with the bacteria. Occasionally transmission to the next generation is effected by an invasion of the arthropod ovary by the bacteria, such as in the case of *Pasteurella tularensis* (McC. & Chap.), which the tick *Dermacentor andersoni* Stiles passes from one generation to the next via the egg.

Several investigators have reported the presence of bacteria in the eggs of insects (*e.g.*, those of mosquitoes and silkworms), but whether or not this indicates that the bacteria are being transmitted to the next generation has not been made clear. It might be mentioned here, parenthetically, that bacteria and other microorganisms may, under certain circumstances, cause the hatching of mosquito eggs that frequently will not hatch in a sterile solution. Some writers believe that this phenomenon is caused by the reduction of dissolved oxygen by the bacteria in the medium; others think that some sort of direct stimulation to hatching is involved.

Sometimes the transmission of bacteria to the next generation of the insect is brought about in a rather complex manner. A case at point is that of the olive fruit fly, *Dacus oleae* (Gmel.). When the eggs of this insect are laid, they pass along the vagina past a perforated membrane, which lies opposite to a small series of bacteria-filled little pouches or pockets in the anal tract. In the process of ovipositing, each egg is pressed against the openings, and bacteria contained in the pouches are smeared over the surface of the egg. The larva that hatches from the egg possesses four spherical caeca near the anterior end of the midgut. The descendants of the bacteria that were passed through the egg are contained in these caeca as well as in the lumen of the alimentary tract. During the pupal stage a bulblike diverticulum branches off the esophagus just in front of the brain. The bacteria accumulate in this structure; from it they spread throughout the gut and into the anal pouches already mentioned. Here they remain until the insect begins laying eggs, at which time they are smeared against the surface of the eggs into which they gain entrance through the micropyle. The details of this process have been worked out by Petri (1910), and other workers have called attention to similar relationships in other Trypetidae.

Bacteria and Nutrition of Insects. Since the alimentary tract of most insects harbors bacteria of some kind, it seems logical to suppose that these

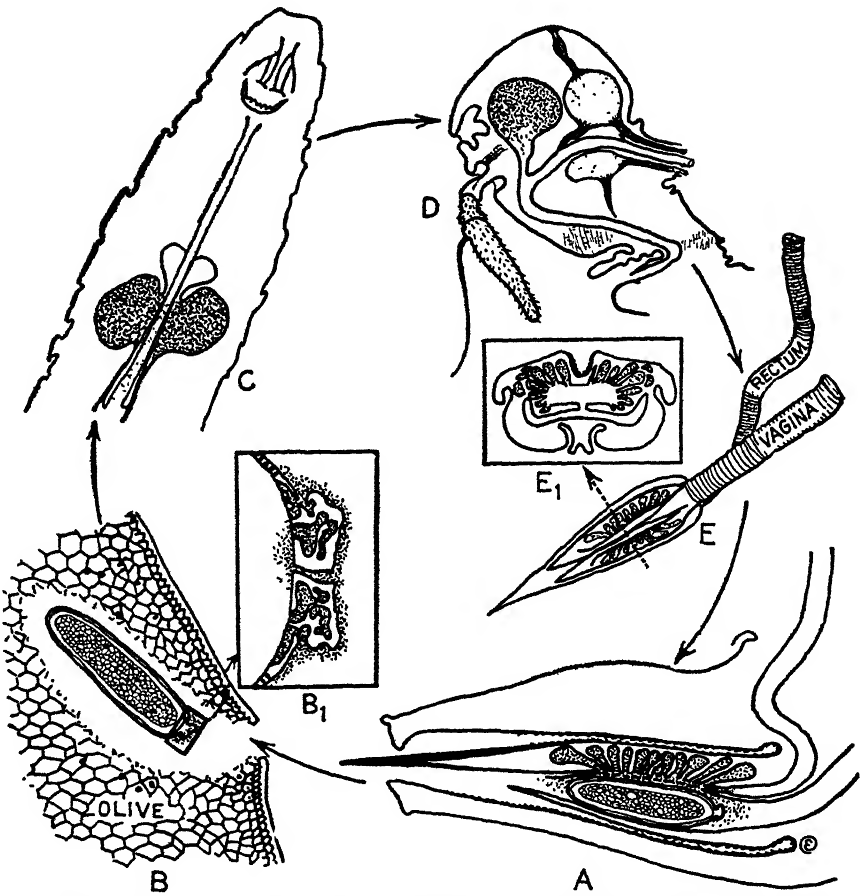


Fig. 35. Transmission of extracellular bacteria in the olive fruit fly, *Dacus oleae* (Gmel.), from one generation to the next. Diagrammatic and not to scale. *A*. A longitudinal section through the ovipositor of the adult fly, showing the bacteria-filled pockets connected to the oviduct by longitudinal slit (dotted line above egg). On passing out, the eggs are pressed against tne pockets and smeared with bacteria. *B*. Egg deposited in an olive fruit. B_1. A section through the micropyle of an egg through which the bacteria enter and "infect" the embryo. The air tubes of the micropyle structure are also filled with bacteria. *C*. Anterior end of mature larva, showing longitudinal section of two of the four spherical caeca (filled with bacteria) near the fore part of the midgut. *D*. Longitudinal section through the head of an adult fly showing the bulbous diverticulum of the esophagus. The diverticulum has retained the bacteria (in all cases indicated by stippling) during the pupal stage. Later in the adult, the entire intestinal tract is recontaminated from this organ. *E*. A view of the female sex apparatus showing the location of the pouches, which have acquired their bacteria from the intestinal tract. E_1. Cross section of pouch area. (*Drawings based on illustrations and description by Petri, 1910.*)

microorganisms exert a marked influence upon the nutritional and digestive processes of the insects concerned.

In the first place, the bacteria may themselves serve as food to microphagous insects. This is seen particularly in the case of mosquito larvae that ingest large numbers of bacteria occurring normally in their environment, and in the case of certain saprophagous insects that feed in or on decaying matter. With some insects, *e.g.*, certain flies, it has been claimed that bacteria are absolutely essential for the growth and development of the arthropods. Others have been reared under sterile conditions. Recent studies have shown that frequently other food substances and certain vitamins may be substituted for the bacteria. Nevertheless, in nature, bacteria and other microorganisms without doubt often serve as a source of food to certain insects.

In addition to being a source of food substances, bacteria may directly influence the mechanics of the host's digestion of its food. This may result, for example, through the liberation of enzymes (proteolytic, saccharolytic, lipolytic, amylolytic, etc.), which bacteria are capable of secreting in large amounts and which may act directly on the food material ingested by the insect. In some cases the bacteria are known to produce enzymes that attack the same substratum as those produced by the insect itself; in other cases the bacteria are the sole source of the enzyme. Some insects, such as the larvae of lamellicorn beetles, have special pouches or "fermentation chambers" in which certain substances, *e.g.*, cellulose, are fermented or broken down so as to make them more easily assimilated by the insect.

The role of extracellular bacteria in synthesizing vitamins and other growth accessory substances in the gut of insects has not been well studied. A sufficient number of observations have been made, however, to indicate that some interesting possibilities in this regard do exist. Thus blowflies appear normally to require the vitamin B factor produced by the bacteria in their intestines. These insects are unable to develop aseptically on sterile blood unless vitamin B in some form is supplied.

Bacteria Pathogenic for Man and Found in Insects. We have already indicated the fact that filth-frequenting insects, such as flies, may carry mechanically on their exterior surfaces such bacteria as those which cause typhoid fever, dysentery, tuberculosis, anthrax, and other diseases. Bacteria causing these and other diseases are also carried within the bodies of certain insects, which act as very efficient vectors. We refer to such bacteria as *Pasteurella pestis*, the cause of plague, which is transmitted by fleas, and *Pasteurella tularensis*, the cause of tularemia, which may be transmitted by the deer fly and by ticks. The insect pathologist must be cognizant of such relationships as these when examining known insect vectors to determine their bacterial flora or to ascertain pathological changes.

For a review and analysis of these relationships the reader may refer to an account of them by the author (Steinhaus, 1946).

Spirochetes

The spirochetes associated with insects and ticks are best known for the disease-producing properties of some of them. Nevertheless, names have been given to approximately 20 nonpathogenic species of spirochetes found in healthy insects. Some of these appear to be truly entomogenous, and some are merely fortuitous associates of their insect host. In addition, a considerable number of unnamed species, both free in the gut lumen and attached to protozoan symbiotes, occur in insects. Most instances of the latter relationship have been observed in termites in which spirochetes are invariably attached to the surface of certain flagellates that live in the gut of the termites. Similar spirochetes have been seen attached to the protozoa living in the gut of the wood-eating roach, *Cryptocercus punctulatus* Scudd.

Undoubtedly many more species of entomogenous spirochetes remain to be discovered. At present, there are no indications that spirochetes are limited to any particular groups of insects. Probably most of the species have so far been observed in mosquitoes and other Diptera, but species have also been reported as occurring freely in the alimentary tracts of cockroaches, fleas, bugs, termites, and other insects.

Of those arthropod-transmitted species which cause disease in man and animals, the most important include those which cause relapsing fever in man, a similar disease in cattle, and septicemia in chickens and geese. The possible presence of these microorganisms must be kept in mind when microbiological or pathological examinations of their vectors are made.

Various types of relapsing fever occur in man, caused by closely related species or varieties of morphologically similar spirochetes. Some strains (*e.g.*, *Borrelia recurrentis* (Sak.)) are transmitted by lice (*Pediculus*); others (*e.g.*, *Borrelia turicatae* (Leb.)), by ticks, mostly species of *Ornithodoros*. Only the ticks transmit the relapsing-fever spirochetes by their bites; transmission with lice occurs when the insects are crushed or otherwise damaged on the skin. The spirochetes in the coelomic fluid of the lice may thus be rubbed into the wounds made by the insects' bites. In both ticks and lice the spirochetes go through definite morphological changes. In the louse, most of them become immobile soon after being ingested and begin to disintegrate, although some penetrate the cytoplasm of the epithelial cells lining the gut. For about 6 days no true spirochetes can be found in the louse, but soon thereafter they begin to appear in the coelomic cavity of the insect where they have been observed to persist for at least 25 days. Apparently different strains of spirochetes vary

considerably in their behavior when inoculated directly into the coelom of lice. The entire cycle of changes that take place in either lice or ticks needs further study before all of its details are known.

Something of the nature of a life cycle has been worked out in the case of *Borrelia anserina*, the cause of fowl septicemia, as it occurs in the tick *Argas persicus* Oken. Various investigators have reported that in the cells of the tick the spirochetes break up into granules. These granules may be introduced into a fowl where they develop into spirochetes again, or they may enter the egg, thus ensuring transmission to the next generation of the tick. A similar type of granule formation supposedly occurs in the case of the relapsing-fever spirochetes in their vectors.

It is thought that a spirochete infection such as yaws may be transmitted mechanically by flies such as those of the genus *Hippelates* and the housefly.

Fungi

The internal extracellular fungi of healthy insects appear to be exceedingly few in number when compared with the numerous species of fungi which parasitize insects or which occur within the tissue cells of insects or which are found primarily on the external surfaces of insects. Occasionally saprophytic fungi (mostly soil and air forms) may be cultivated in considerable numbers from the alimentary tracts of herbivorous insects. In most of these instances the relationship is merely a fortuitous one. Only rarely are fungal spores found in insects that suck blood or plant juices.

As an external relationship, the fungi which are specially cultivated and tended by such insects as ambrosia beetles, termites, and certain ants and which serve as food for these insects, have already been considered. Obviously, when such fungi are ingested, the association becomes an internal one. In such cases, however, reproductive units of the fungi are unlikely to be present since, as we have seen, the fungi cultivated for food are usually eaten while the plants are in an immature and tender condition. These fungi probably are not to be considered as part of the natural flora of the insect gut.

On frequent occasions, when the character of the intestinal flora is being determined, one isolates a yeast or yeastlike organism which may be present in considerable numbers in the gut contents of the insect. Such yeasts are usually the adventitious saprophytic type. Sometimes they represent the fulfillment of the food requirements of the insect, which depends upon yeasts for its nourishment. Under natural conditions the larvae of drosophila flies, for example, feed principally on yeasts and other microorganisms. Microbe-free larvae grow rapidly on sterile food but die before pupating. If dead yeast is supplied for their nourishment they

develop quite as well as on living yeast—both forms contain the essential factors. These may also be supplied by bacteria and fungi, but yeast appears to constitute a more complete food. The yeasts ingested by these insects flourish on the decaying fruit frequented by the flies, which are saprophagous. The insects may gain some nutriment directly from the fruit itself, but the main role of the fruit is that of providing a substratum for the growing yeast cells which are ingested by the larvae. Although not specifically required by them, some insects, such as certain mosquito larvae, are able to obtain all their nutritional requirements from yeasts alone.

To be remembered is the fact that some of the yeasts and other fungi found within the alimentary tracts of insects are the cause of specific diseases of certain plants. For example, at least 11 species of insects (*e.g.*, species of *Nezara* and *Dysdercus*) are known to be vectors of yeasts of the genus *Nematospora* which cause such diseases as yeast spot of lima beans, dry rot of citrus fruits, and others. Yeasts that cause souring of figs have been isolated from insects frequenting this fruit.

Some of the true fungi causing extremely destructive diseases of plants are found both externally and internally associated with their arthropod vectors. Thus *Ceratostomella ulmi* (Sch.), the cause of Dutch elm disease, is so associated with its vectors, which include certain bark beetles (*Scolytus*) and possibly mites. The same holds for the species of *Ceratostomella* which cause blue stain of conifers and which are carried by beetles of the genera *Glischrochilus* and *Dendroctonus*. Other disease-producing fungi might be cited. The important thing for the insect pathologist to bear in mind when examining the flora of an insect is that such fungi may be present in the insect in large numbers and that their significance in this location must be accurately determined if a proper perspective of the microbiological relationships involved is to be gained.

Viruses

Those viruses present in insects but not producing disease in the insects themselves either are agents of human, animal, and plant diseases or are elements known as "bacteriophages," which cause infection and destruction of bacteria. Since we are concerned here only with the microbial life of healthy insects, the first category, *i.e.*, those pathogenic for insects, will be reserved for full treatment in a later chapter.

The exact location of a virus with respect to the cells of its arthropod vector is in most instances virtually unknown. Since viruses characteristically multiply and increase only within living cells, it seems likely that the association is an intracellular one in all those cases in which the virus increases quantitatively while within the body of its arthropod vector.

On the other hand, in those instances in which the body of the insect or tick is merely a temporary conveyance for the virus and in which there is a decrease or at least no quantitative increase in the amount of virus present, it is possible that the virus survives in an extracellular relationship.

If we were to keep strictly within the limits of the category of insect-microbial relationships we are discussing, namely, the extracellular internal microbiota, we should probably have to make only a token mention of viruses here because of their supposed intracellular location. For the sake of convenience, however, the few statements made here will be presented without too much regard for the exact location of these agents with respect to the cells of their insect hosts. Since many viruses attacking animal and plant cells reside, while in their vectors, principally in the fluids of the insect, considering them as extracellular agents or as intracellular cytotropes has about equal merit in many cases.

Plant-disease Viruses in Insects. Most of the viruses transmitted by insects cause diseases of plants. For a thorough coverage of these particular viruses, the reader should consult such books as those by Bawden (1939) and Leach (1940).

The plant-disease viruses are carried by insects of several orders, the principal ones of which are Orthoptera, Thysanoptera, Homoptera, Hemiptera, and Coleoptera. As far as is known, none of the insects included in these groups appear to be adversely affected by the presence of the viruses in their bodies. Accordingly, these viruses rarely, if ever, complicate the picture when one is dealing with a disease of the insect itself. At least gross changes are not readily apparent. Minute histopathological changes may occur in viruliferous insects which one would have to differentiate from the histopathology of a true disease of the insect itself.

As has been mentioned earlier, a plant virus is sometimes found on the exterior of an insect that may or may not be in the process of transmitting the virus to susceptible plants merely by carrying the virus as a contaminant on its external parts. Or the virus may be mechanically transmitted by passing through the insect's body but without undergoing any multiplication or developmental changes in so doing. Or the virus may actually multiply or otherwise develop within the body of the insect; if the virus is then carried to a susceptible plant, the process sometimes known as a "true biological transmission" occurs. Other ways of classifying the methods of insect transmission have been suggested, such as those based upon the length of time necessary before an insect is capable of transmitting the virus and those based upon the group of insects concerned (aphids, leafhoppers, thrips, etc.). Whatever the method of grouping the types of transmission, it should be remembered that the

location of the virus within the insect is likely to vary with the particular manner by which the virus is transmitted to plants. As concerns the main reservoir of virus in the infective insect, the blood is probably most important in this regard. From the blood, the virus probably passes slowly into the salivary glands in those instances in which transmission occurs during the time the insect feeds. Very little virus is lost by excretion with the feces.

In the case of certain of the viruses transmitted by aphids, *e.g.*, the virus of sugar-cane mosaic transmitted by *Aphis maidis* (Fitch), the virus apparently is in the insect's salivary glands from which it is liberated along with the abundant flow of saliva into the tissues. *Myzus persicae* (Sulz.), the vector of potato leaf roll and numerous other viruses, also probably carries virus in its salivary glands. The virus causing curly top of sugar beets and other plants has been reported from the salivary glands, hemolymph, feces, and alimentary tract of the leafhopper *Eutettix tenellus* (Bak.). The principal reservoir appears to be the hemolymph. Although the virus overwinters without change in virulence in the leafhopper, there probably is no multiplication of the virus within the insect. In the case of the virus causing streak of corn, it is thought that the virus enters the alimentary tract of its vector, *Cicadulina mbila* (China), through the mouth, passes through the intestinal wall into the blood and then into the salivary gland, from which it may be introduced into the plant during the feeding process. Sometimes the virus is unable to penetrate the intestinal wall, and in such cases the insects are unable to transmit the virus. It is interesting that although the virus may be detected in the rectal contents of the infected leafhopper, it is not present in naturally voided feces.

The virus may remain in its vector for the rest of the insect's life, or it may be retained for a short time only. Sometimes both of these possibilities occur with one virus in a single host species; thus the virus of aster yellows may remain with its leafhopper vector (*Macrosteles divisus* Uehler) until the insect dies, or it may be lost within a very short time. It is possible that the temperature of warm summer months may cause the leafhoppers to lose their virus. A virus may be present in and transmitted by the nymphal as well as the adult stages of its vector, *e.g.*, peach yellows and *Macropsis trimaculata* (Fitch). Some insects are unable to acquire virus in the adult stage but acquire it only in an immature stage, after which it may be transmitted either when the insect is in the nymphal stage or when it is an adult. Such is the case with the virus of yellow spot of pineapple transmitted by *Thrips tabaci* Lindeman. An instance of the transovarial transmission of a plant virus from one generation of the insect to the next is that of the virus of dwarf disease of rice which passes

through the egg of *Nephotettix apicalis* var. *cincticepts* Uehler. This plant virus, incidentally, was the first one shown to be transmitted by insects—a discovery of a number of Japanese investigators working independently between 1901 and 1909.

Animal-disease Viruses in Insects. Many viruses pathogenic for man and animals, and transmitted by insects, are well known to most readers of this book. Only rarely will it be necessary for the insect pathologist to differentiate such viruses from other microbiota of the insect with which he may be concerned. As with the plant viruses, however, it may be necessary to keep the possible presence of the animal viruses in mind when studying the details of histopathology and cytological changes brought about in insect tissues. It is entirely possible that the presence of plant and animal viruses may cause slight but distinct changes in the appearance of such cellular structures as the nucleus and mitochondria which would confuse the histopathological picture brought about by an entomogenous pathogen.

One of the best known examples of a distinct biological relation between an animal virus and insects is that of the virus of yellow fever and mosquitoes, mostly of the genus *Aëdes*. There appears to be no significantly harmful effect caused by the virus on the mosquito. Neither its longevity nor any of its life processes seem to be adversely affected, even though the insect's tissues retain the virus for the remainder of the mosquito's life. The limit of the virus distribution in the body of the insect is not clear. The presence of the virus has been demonstrated in the head, thorax, and abdomen of mosquitoes before their bites were infective. The legs, midgut, hindgut, ovaries, salivary glands, and occasionally the feces of infected mosquitoes have all been reported as containing yellow-fever virus, but none has been demonstrated in the hemolymph or in the mouthparts. Although some authorities believe that the virus multiplies within the mosquito, others think that it does not since shortly after the insect has an infective blood meal the quantity of virus in the insect decreases rather than increases. The delay required before the mosquito's bite becomes infectious represents the time necessary for the virus to reach the salivary glands by simple mechanical transportation. Some workers have presented evidence to show that, although the virus content of a mosquito is lowered for a few days after an infective feeding, after the first week the quantity rapidly increases until more virus is present than that originally ingested. Whitman (1937), who supports this idea, has written that the maximal titer obtainable after incubation might be lower than the artificially high titer apparent following the ingestion of the fully virulent blood of the monkey used for experimental infection. Beyond a certain point the growth requirements of the virus might surpass

the supporting ability of the mosquito's cells. Thus there would be a much greater supply than demand. A true life cycle of the virus in mosquitoes apparently does not exist. Each time an infective mosquito bites its host, at least 100 infective doses of virus are injected, and this is probably equivalent to about 1 per cent of the insect's total virus content. Although experimentally the virus fed to larvae will persist through to the adult stage, there is no evidence that the virus will pass through the egg from one generation of the mosquito to the next.

As a disease, dengue is similar to yellow fever in that the virus agent is transmitted by mosquitoes, principally *Aëdes aegypti* (Linn.). An incubation period of 8 to 11 days is necessary before the mosquitoes can transmit the virus. There is some evidence that the virus may multiply in its insect hosts. Infected mosquitoes have been known to remain alive for 200 days and to remain infective as long as the temperature remains above 18°C. As with the virus of yellow fever, there is no indication that the virus of dengue passes through the egg of the mosquito.

Of considerable interest in recent years is the relation between insects and the viruses responsible for encephalitis and related infections such as St. Louis encephalitis, equine encephalomyelitis, Japanese B encephalitis, and Russian spring-summer encephalitis. The virus of the last-named disease is transmitted by ticks, but the others are transmitted principally by mosquitoes (*Aëdes*, *Culex*, and others). Some authorities believe that the transmission of the equine and other strains is not mechanical but occurs after multiplication, maturation and, less probably, after cyclic changes of the virus within the mosquito. The virus may be located widely throughout the body of the insect, including the legs, head, thorax, abdomen, and body fluid. Such general distribution, however, does not appear to harm the mosquito in any way. Transovarial transmission apparently does not occur. Experimental transmission of the virus of eastern equine encephalomyelitis has been obtained with practically all species of *Aëdes* tried. This is in contrast to species of the genera *Culex*, *Mansonia*, and *Anopheles* which are not capable of transmitting the virus or are not efficient vectors. In nature the relative importance of the various species of *Aëdes* is determined by ecological factors. Several types of encephalitis virus have been isolated from chicken mites, and domestic fowl appear to be an important vertebrate reservoir of the virus.

Numerous other viruses pathogenic for man and animals are known to be associated with insects and other arthropods. If viruses could be seen and cultivated as easily as most bacteria, the insect microbiologist and insect pathologist would undoubtedly record their presence in specimens with which he worked much more frequently. Although, for the most part, viruses affecting vertebrates cause no easily recognizable pathology

in insects, thorough investigation of this point has been so superficial that, as we have already mentioned, the insect pathologist must not overlook this possibility when he is concerned with the detection of minute pathological changes in insect tissue.

Bacteriophage. Only a few instances have been recorded in which those ultramicroscopic agents known as "bacteriophages" have been recovered from insects. One of the discoverers of bacteriophage, d'Herelle, is supposed to have first noticed the effect of this lytic agent in 1909 in cultures of "*Coccobacillus acridiorum*," the bacterium responsible for an epizootic disease of locusts in Mexico. Bacteriophages attack and destroy bacteria for which they frequently have a specific affinity. Sometimes the bacteriophage may be isolated from the insect along with its respective bacterial species; at other times extracts must be carefully made from triturated insects. This latter method was used when a bacteriophage was first isolated from an insect, the housefly, *Musca domestica* Linn., by Shope (1927). In this case when a salt-solution extract was made of houseflies, it yielded a bacteriophage active against four strains of bacteria (*Salmonella typhosa* (Zopf), *Salmonella paratyphi* (Kay.), *Escherichia coli* (Mig.), and *Micrococcus muscae* (Glaser) [*Staphylococcus*]). Glaser (1938) found that houseflies caught in nature or bred in a contaminated, *i.e.*, nonsterile, state invariably harbored bacteriophage.

Other bacteriophages have been isolated from insects as well as from ticks. These include one against the plague bacillus, *Pasteurella pestis*, (L. & H.) from fleas (*Xenopsylla cheopis* (Roth.)), one from the tick *Dermacentor andersoni* Stiles against a micrococcus, and one against a gram-negative small rod from *Dermacentor albipictus* Pack.

The actual role, if any, of bacteriophage within the gut of insects or its effect on the bacterial flora of insects is unknown.

Protozoa

Even excluding those species of protozoa which parasitize insects or cause diseases in them, the protozoan fauna of insects is both large and varied. With few exceptions, very little is known concerning the biological relationships between the protozoa and their insect hosts. In such cases as those concerned with the flagellates of termites, and certain of the human pathogens and their vectors, the biological relationships are fairly well known. This, however, does not apply to the vast majority of protozoa found in insects. Most of the recorded species have been dealt with almost entirely from a systematic viewpoint only.

Most of the protozoa found in ticks are parasites of other animals also, and in general the protozoan fauna of arachnids is meager as compared with that of the Hexopoda. Of the latter group almost every order contains

members that maintain an association of one sort or another with protozoa. These protozoa, in turn, are representatives of all five classes: Mastigophora, Sarcodina, Sporozoa, Ciliata, and Suctoria, although members of the last-named class are rarely found in insects. For our present discussion, limited to those species associated only with healthy insects, it is perhaps preferable to treat our subject according to the five classes of protozoa just mentioned. Those protozoa (mostly Sporozoa) which cause infections in insects will be covered in a later chapter.

Mastigophora
(Flagellata)

Most of the Mastigophora, or flagellates, associated with insects live in the alimentary tracts of these arthropods. For the sake of convenience we might consider them according to the categories devised by Becker (1930): (1) those flagellates which live in the intestines of certain termites; (2) those which belong to the family Trypanosomidae and which are exclusively entomogenous, having no definite vertebrate or plant host; (3) those which belong to the family Trypanosomidae and which spend a part of their life cycle in the intestines of insects and the remainder in the blood stream or tissues of vertebrates; and (4) all intestinal flagellates of insects belonging to families other than Trypanosomidae.

Flagellates in Termites. Apparently the first man to notice the rich protozoan fauna of termites was Lespes in 1856. Soon thereafter, other workers, notably Leidy and Grassi, made similar observations until now well over 500 species of termites have been studied with respect to their protozoan fauna and approximately 300 species of protozoa have been named and described from these hosts. Contributing to this valuable accumulation of data, and to the biological relationships involved, have been the writings of several modern workers, including those of Cleveland, Hungate, and especially those of Kirby.

Most of the flagellates occurring in termites are included in the orders Polymastigida and Hypermastigida, the two highest orders of the class Mastigophora, with the number of Polymastigida about twice that of Hypermastigida. For a listing of the names of various species of termite protozoa and their hosts, the reader is referred to a list of these which has been presented by the author (Steinhaus, 1946). Parenthetically, it might be mentioned here that the wood-eating roach, *Cryptocercus punctulatus* Scudd., characteristically has a fauna of protozoa similar to that found in termites.

Although exceptions do exist, as a general rule all the termites of a given species have identical faunas. Many species of protozoa are found in only one host, but certain species occur in several hosts. Many species

of termite flagellates have a present host distribution that indicates a greater stability in the characteristics of the protozoa than has existed during the same period of time in the insects. Speciation has occurred in the termites without having taken place in certain of their protozoa, and it seems probable that many of the flagellate types known today existed in the ancestors of the termites (Kirby, 1937, 1941).

Transmission of the protozoa from termite to termite is thought to take place directly from one termite to the other. One means by which this is probably accomplished is through the receiving of proctodaeal food from the anus of the adult termite by the young termite. At any rate, intimate contact appears to be necessary since refaunation must take place following each molt (as well as at the beginning of the insect's life) and since without the intimate contact no refaunation takes place and the termite dies.

Of particular interest are the physiological aspects concerned in the mutual relationship between the termite host and its protozoan fauna. Cleveland (1926) has developed methods of freeing the termites from their flagellates and for studying the role of the protozoa in the physiology of the termites' nutrition. Defaunation may be accomplished by incubating the termites at relatively high temperatures (36°C. for 24 hours), by subjecting them to starvation which kills the flagellates before the termites succumb, or by placing the termites in oxygen under pressure, which destroys the protozoa but leaves the termites unharmed. By these methods it was conclusively demonstrated that the flagellates are absolutely necessary for the termite to maintain its life for any extended length of time.

Most termites feed exclusively on wood; the protozoa in the gut of the insect take into their bodies the tiny particles of wood which, under more or less anaerobic conditions, they literally digest, thus making available to the termite the cellulose contained therein. When the protozoa are removed from a wood-feeding termite, the insect is unable to derive nutrients from its food, and it dies. When defaunated termites are fed a diet of cellulose products, they apparently suffer no nutritional lack and carry on their life processes the same as do normal termites possessing their regular fauna. It appears rather clear that, along with other products, glucose is formed from the cellulose by the termite protozoa. Just how the glucose is made available to the termite, though, has been open to question. One theory (Hungate, 1939) postulates that the glucose produced by the digestion of cellulose is retained within the bodies of the protozoa and undergoes a dissimilation that yields energy for their life processes. Presumably the products of the fermentation of the glucose by the protozoa would be oxidized by the termite to carbon dioxide and water. Other theories have been proposed.

An interesting feature of the relation between the *Cryptocercus* roach and its protozoa has to do with the reaction of these two forms of life to the same hormone. Cleveland (1947) has shown that while in the roach the hormone produces molting, in the protozoa it produces several types of sexual behavior, some of which appear to be significant from the standpoint of the origin and evolution of sexual processes in general.

Entomogenous Trypanosomidae. Of the six genera of Trypanosomidae, *Trypanosoma*, *Leishmania*, *Phytomonas*, *Crithidia*, *Leptomonas*, and *Herpetomonas*, the last three are associated exclusively with invertebrates,

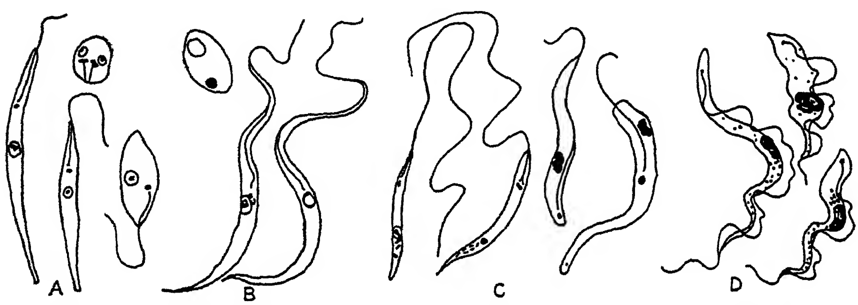


Fig. 36. Representative Trypanosomidae found in arthropods. *A. Leptomonas ctenocephali* Fantham from the gut of the dog flea. *B. Crithidia hyalommae* O'Farrell from the body cavity of a tick (*Hyalomma*). *C. Herpetomonas muscarum* (Leidy) from the gut of the housefly. *D. Trypanosoma gambiense* Dutton, the cause of African sleeping sickness, transmitted by tsetse flies. (All figures diagrammatic.)

particularly insects. Those species which have both vertebrate and invertebrate hosts are included in the genera *Trypanosoma* and *Leishmania*; members of the genus *Phytomonas* have both an invertebrate and a plant host.

Although some authorities consider *Leptomonas* and *Herpetomonas* to be cogeneric, for our purposes it is more convenient to treat them as separate genera. Thus those flagellates which have only leptomonad and leishmania forms are considered as *Leptomonas* and those which in addition have the crithidial and trypanosome forms as *Herpetomonas*.

Leptomonas ctenocephali is a noteworthy example of the genus *Leptomonas*, and it lives in the intestinal tract and Malpighian tubes of the dog flea, *Ctenocephalides canis* (Curtis). If one examines the epithelial lining of such a flea, one will observe that it is covered with a mosaic of short stubby flagellates, which have attached themselves to the lining by their anterior ends. Some flagellates remain free in the gut and pass out with the insect's feces. In addition, small ovoid leishmania forms covered with a cystlike wall may be seen in the contents of the posterior end of the gut. The feces of the adult flea are fed on by the larva, which thus acquires

the flagellates that survive throughout the pupal to the adult stage. This flagellate is cultivable on special blood media (*e.g.*, N.N.N. medium). Other species of *Leptomonas* have been observed in other fleas, in mosquitoes, certain bugs, roaches, and in insects of agricultural importance. An instance of the latter is *Leptomonas pyraustae*, which Paillot found living in the midgut of the European corn borer, *Pyrausta nubilalis* (Hbn.), in France (see page 551).

Herpetomonas muscarum is a typical example of the form of flagellates grouped in the genus *Herpetomonas*. It is frequently unnecessary to look very far to find this species. One has but to make a wet-mount slide of the gut contents of a housefly, *Musca domestica* Linn., and the chances are good that when the slide is examined under a microscope, large numbers of agile twisting flagellates will be seen swimming rapidly about among the detritus of the insect's intestine. Especially is one likely to see this flagellate in flies collected in the tropics. The housefly apparently is not the only host of the protozoan, since this flagellate or closely related species has been reported from several other species and genera of flies. *H. muscarum* may be found in any part of the fly's alimentary tract posterior to the proventriculus. It is usually seen in the leptomonad form, having a slender body with a size of 30 by 2 to 3 microns. Transmission usually takes place through the agency of cysts, which are eliminated with the feces of the contaminated flies. The flagellate is cultivable on artificial media containing a high concentration of blood. Other species of *Herpetomonas* have been observed in such insects as drosophila flies, mosquitoes, hornets, fleas, and certain bugs.

A large number of *Crithidia* have been reported from insects. Of the better known species mention might be made of *Crithidia fasciculata* from the gut of *Anopheles maculipennis* Meig., and *Crithidia gerridis* from the intestine of certain water bugs, including *Limnogonus fossarum* (Fabr.). In the latter case, the encysted protozoan is ingested by the insect host, in which it soon develops flagella and begins to multiply by binary fission. Characteristic groups, or rosettes, of protozoa are formed which attach themselves to the insect's intestinal epithelium. These groups gradually break up, and the elongated crithidial forms swim away and eventually multiply again.

Trypanosomidae Having Both Invertebrate and Vertebrate or Plant Hosts. Here we are concerned with the genera *Leishmania*, *Trypanosoma*, and *Phytomonas*. The members of the last-named genus all have plant hosts; otherwise they are entirely similar to members of the genus *Leptomonas*. The genus *Leishmania* is particularly important because of its three members (*L. donovoni*, *L. tropica*, and *L. brasiliensis*) which cause the tropical diseases of man known as "kala azar," "oriental sore," and

"espundia." The relation of insects to the transmission of these diseases is not at all clear, but sand flies (*Phlebotomus*) have been strongly incriminated.

The genus *Trypanosoma* has had a considerable amount of attention directed to it because of the importance of at least three of its disease-producing species: *Trypanosoma gambiense*, the cause of central and west African sleeping sickness, transmitted by the tsetse fly, *Glossina palpalis* (Rob.-Desv.), and other species of *Glossina; Trypanosoma rhodesiense* (a strain of *T. brucei*), the etiological agent of east and south African sleeping sickness, transmitted by species of *Glossina;* and *Trypanosoma cruzi*, the cause of Chagas' disease in South America, transmitted by species of bloodsucking reduviids of the genera *Triatoma* and *Rhodnius*. In addition, there are numerous trypanosomes (*e.g.*, *T. lewisi*, found in rats and transmitted by fleas) which cause diseases of animals and which have insect vectors. Most of the trypanosomes undergo a more or less characteristic developmental cycle in their insect hosts. This includes the development of several morphological types in the various parts of the insect's alimentary tract and a migration of the flagellates to the salivary glands of the insect where, as in the case of African sleeping sickness, they assume a form capable of infecting a vertebrate host or, as in the case of *T. cruzi*, the feces of the vector becomes filled with trypanosomes that are infective when rubbed into the wound caused by the insect's bite or into mucous membranes of the eyes and mouth.

Entomogenous Flagellates Other than Trypanosomidae. This is the last of the four groups designated by Becker (1930) as containing those flagellates associated with insects; it includes the species other than those in the family Trypanosomidae (order Protomonadida). Remaining in the order Protomonadida are a few entomogenous species belonging to genera in other families. Examples are several species of *Retortamonas* found in mole crickets, cockroaches, water bugs, certain beetles (such as *Retortamonas phyllophagae* in the Japanese beetle and other Scarabaeoidea), and in certain Trichoptera.

The orders Polymastigida and Hypermastigida are large ones and have already been considered in our mention of the flagellates associated with termites. Flagellates of these orders, however, occur in insects other than termites. Thus, of the polymastigotes, species of *Polymastix* and *Monocercomonas* occur in certain beetles, *Tetratrichomastix* and *Hexamita* in roaches, *Eutrichomastix* in caddis-fly larvae; species of hypermastigotes have been observed in roaches.

Only a few species of Rhizomastigida have been reported from insects. An example is *Rhizomastix gracilis*, found in the intestinal tract of tipulid larvae.

Other Classes

Probably most of the protozoa which are associated with insects but which do not cause true infections in them are included in the class Mastigophora. Most of the entomogenous protozoa in the other four classes cause infection or disease in insects. These protozoa are considered in detail in Chap. 12. At this point we shall mention some of those nonpathogenic protozoa which are members of classes other than Mastigophora.

Sarcodina. Some species of amoebae are pathogenic for insects, and these will be discussed later. Healthy insects may also harbor amoebae, however, and occasionally insects have been found to act as carriers of amoebae pathogenic for man. Thus the cysts of *Entamoeba histolytica*, the cause of amoebic dysentery, are known to be able to survive passage through the alimentary tract of cockroaches and houseflies and to be viable in the feces of these insects.

Other amoebae have been found associated with cockroaches. Several species of *Endamoeba* and at least one species of *Endolimax* have been observed in these insects.

Amoebae have also been reported from *Chironomus* larvae, from the Japanese beetle, and from tipulid larvae. The species from *Chironomus* larvae, *Amoeba chironomi*, is distributed throughout practically the entire digestive tract of the insect. It is highly sensitive to environmental changes and encysts rapidly. This is an opportune place to point out that the presence of a contractile vacuole is very rare in the amoebae that are parasitic; but in nonpathogenic amoebae, such as *A. chironomi*, a contractile vacuole is usually present.

Sporozoa. Since all members of the class Sporozoa are parasitic in habitat and since the sporozoan infections in insects usually produce distinct and characteristic pathologies, these protozoa will be treated in detail in the chapter on the protozoan infections in insects. To be sure, since some Sporozoa, such as the gregarines, live in their insect host as rather benign parasites or as commensals, they could perhaps be discussed here as a part of the fauna of healthy insects. However, since even the gregarines invade and destroy certain tissue cells of their hosts, it seems proper to consider them under the category of infections. Even the species of Sporozoa which produce disease in other animals and which are transmitted by insects infect the tissues of their arthropod vectors and bring about pathological changes in them.

Ciliata. Of the class Ciliata, three orders, Holotricha, Spirotricha, and Peritricha, contain entomogenous species. Some of these are pathogenic for their hosts. Others occur in the alimentary tracts of healthy insects,

including those of certain cockroaches and water insects. Ciliates of the genera *Balantidium* and *Nyctotherus* are particularly prominent in this connection. The association appears to be essentially that of commensalism.

Suctoria. Protozoa of the class Suctoria are not known to associate themselves with insects internally except in rare instances. They are usually found attached to the outside surface of the insect and may occur along with other attached protozoa. They may be found within the gut of water-inhabiting insects as the result of being ingested along with food and water.

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CHAPTER 5

INTRACELLULAR MICROBIOTA

By the middle of the nineteenth century zoologists and entomologists, prompted by the availability of more suitable microscopic equipment, were beginning to satisfy their curiosity as to the internal structures of insects. To be sure, the prominent organs and structures of an insect's body were relatively well known at that time but numerous small more concealed structures had been generally overlooked. Even more important was the fact that the functions of some organs and tissues were completely unknown or only guessed.

Among these anatomical parts which defied explanation as to their function were certain tissues and small organlike structures of various shapes and sizes located in various parts of the insect's body. First examinations of these tissues revealed in them no contents of particular significance, and vague and indefinite functions were assigned to them. This was the situation in 1850 when the cytologist Leydig described these peculiar organs in aphids, and when such men as Huxley (1858), Balbiani (1866–1871), and Tannreuther (1907) dealt with these structures in their accounts on the Aphididae. The tissues to which we refer are known today as "mycetomes" but previously have been called "pseudovitelli," "green bodies," and "symbiotic organs."

About the same time that the mycetome of the aphids was discovered and described, peculiar microscopic bodies were found in the hemolymph and tissues of the scale insects. Leydig (1850, 1854), working with *Lecanium hesperidum* Burm., observed in the hemolymph peculiar small lanceolate bodies that multiplied by budding. Leydig did not realize the significance of these bodies, nor did Putnam (1880) later, when he noticed funguslike objects in the scale insect *Pulvinaria innumerabilis* Rath. In 1886, Blochmann reported the presence of bacteriumlike bodies in the follicular membranes and in the eggs of ants and wasps. He became fairly well convinced of the bacterial nature of these minute objects after he (1887, 1888) had seen similar bodies in the fat tissue and eggs of cockroaches. His observations were confirmed by several workers whose reports immediately followed.

Ultimately it was realized that these peculiar yeastlike and bacteriumlike organisms were analogous to similar bodies that were being seen within

the pseudovitelli, or, as we know them, the mycetomes. Krassilstschik (1889, 1890), Henneguy (1904), Pierantoni (1909, 1910*a*,*b*), Uichanco (1924), and others, verified the fact that the mycetomes of aphids contained microorganisms. Šulc (1910*a*,*b*) extended these observations to additional Homoptera; and other workers to roaches, beetles, bugs, ants, and other insects. The entire subject was placed on a firm footing by Buchner and his students (*e.g.*, Stammer and Koch) who published revealing reports between the year 1912 and the present. This group of workers convincingly demonstrated the morphological distinctiveness of the mycetome and the organismal nature of the bodies contained within this structure. Buchner (1930, 1939, 1940) has presented summaries of his own observations, as well as digests of the findings of others, and these should be consulted by every interested student. Additional reviews of the subject include those by Glaser (1930*c*), Paillot (1933), Beier (1938*a*,*b*,), Baumgärtel (1940), and Steinhaus (1940, 1946). The interested reader is urged to consult these reviews and other publications for detailed information concerning insect-microbe symbiosis, since only a brief mention of this interesting relationship can be made in the present volume.

Fig. 37. Paul Buchner, German scientist well known for his monumental work and publications on intracellular symbiosis.

SYMBIOTES, MYCETOCYTES, AND MYCETOMES

The insect pathologist cannot afford to limit his knowledge of insect microbial flora and fauna to that of the extracellular microbiota only; the intracellular microbiota is also extremely important to the student of insect diseases. The reasons for this will become obvious as the reader proceeds through this book. Suffice it to say here that such knowledge is made necessary if for no other reason than to ascertain the validity of the frequent assertion that these intracellular forms may, under certain conditions, affect the host so as to cause it to become diseased.

It behooves us, therefore, to inquire into the nature of such things as symbiotes, mycetocytes, and mycetomes and to know something of their function in the life of their insect hosts.

The Terms "Symbiosis" and "Symbiote." The use of the term "symbiosis" has been subject to considerable abuse by biologists and nonbiologists alike. De Bary (1879) originally used the word to denote simply the living together of dissimilar organisms regardless of what might be the result of such an association. Subsequent authors have frequently construed the meaning of the word to include only those unions in which the association was mutually advantageous. Such usage does not follow that which De Bary originally intended it to have nor is it in accord with the recommendation of the Committee on Terminology of the American Society of Parasitologists (*J. Parasitol.*, 1937, **23**, 326–329). The term "symbiosis" is a broad one including not only the relationship of mutualism (the relationship in which the association is mutually advantageous), but parasitism and commensalism as well. It will be so used in this book.

Keeping in mind De Bary's meaning of the word "symbiosis," we turn now to the correct usage of the word "symbiote," meaning simply an organism that lives in association with another organism. "Symbiont" is the form originally coined by De Bary, and it is used by many writers. Wide usage probably makes it an acceptable form except that it is poorly formed etymologically. The word is derived from the Greek *sumbiotes*, meaning "companion," "partner," "one who lives with." Accordingly, the correct English form is "symbiote," since "symbiont" really has no Greek original. These facts have been recognized by the Committee on Terminology, but a definite opinion on the word's usage has not been rendered. In our use of the word we shall accept the preference of the philologist and use the form "symbiote." Since it refers to those microorganisms associated either extracellularly or intracellularly with insects, we shall consider the word to mean broadly any microorganism that lives in association with an insect; more especially we shall use it in referring to a microorganism that lives in an intimate and rather constant or continuous association with its host.

The term "symbiote" may properly be used for either member of a symbiotic association. It is customary, however, to refer to the smaller member as the "symbiote" and to the larger member as the "host." To avoid confusion, the term "microsymbiote" is sometimes used to designate the smaller symbiote, especially when the latter is a microorganism. Furthermore, the term "endosymbiote" is sometimes employed in referring to those symbiotes which live intracellularly, and the word "exosymbiote" to those which live extracellularly.

An expression that in many instances is synonymous with the word "symbiote" is the term "cytotrope" or "cytotropic agent." Moshkovsky (1945), for example, uses these words in referring to those microorganisms and viruses which live in an intimate and obligatory relationship with the

cells of their hosts. It is apparent that the word "symbiote" has a broader meaning than this since, as in the case of the microorganisms associated with insects, it includes all types of associations—even those in which the union is not obligatory. Presumably we might refer to an intracellular microorganism as a "cytotrope" as long as it remains in a relationship with its host that is obligatory. If, however, the microorganism were cultivated on artificial media, the obligatory relationship would be broken down and the term "cytotrope" would not apply according to Moshkovsky's definition. Such examples do exist.

The Nature of Intracellular Symbiotes. Any doubt that microorganisms could live harmlessly or mutualistically within the tissue cells of other living organisms was removed by the work of Beijerinck and others who, in the latter part of the nineteenth century, showed that the rodlike elements in the root tissues of certain plants were in fact cultivable bacteria. Unlike these symbiotes in leguminous plants, most of those in insects have not been cultivated apart from their hosts. Indeed, their cultivation remains the greatest need in order to have a real understanding of these microorganisms.

Cultivation on artificial media has been obtained with certain of the rickettsiae or rickettsialike organisms and possibly with some other bacterial symbiotes such as those in the fat body of cockroaches. Nearly all species of pathogenic rickettsiae have been grown in tissue cultures, particularly in the tissues of the embryonic chick. It is probable that many of the mutualistic symbiotes in insects could be cultivated by similar special techniques.

When methods of growing the majority of the intracellular symbiotes away from their hosts are developed, more progress may be expected in determining the true nature of these microorganisms. Although some of the intracellular forms are certainly bacteria and others definitely appear to be yeasts, the identity of others is entirely uncertain. We shall attempt later in this chapter to elucidate the points of difference by discussing the intracellular symbiotes under three large headings: Bacteria, Rickettsiae, and Yeasts.

Although in some respects morphologically similar, the intracellular symbiotes and the mitochondria appear to be distinctly different types of cell inclusions. A few cytologists believe that mitochondria are bacterial in nature, but most authorities distinguish these bodies from true microorganisms by staining variations and differences in response to certain solvents and other chemicals. In most cases, the general appearance of the two and the particular "picture" they present are distinct enough to permit one to detect the difference readily. In the mycetocytes of some insects the mitochondria may be seen lying beside and among the symbi-

otes. The belief that the symbiotes represent waste products of cellular metabolism has now been abandoned. That they are actually distinct entities, separate from, and foreign to, the tissues of their hosts has been shown, in the case of cockroaches, by serological tests. Complement-fixation tests, for example, give positive reactions in homologous combinations only; *i.e.*, symbiote antigen combines only with symbiote antiserum and host-tissue antigen only with host-tissue antiserum; the two do not cross-react.

Origin and Role of Intracellular Symbiotes. On the basis of our present knowledge, the origin of the intracellular symbiotes in insects can only be guessed. One assumption might be that in their initial association these microorganisms were pathogenic parasites and that then as the process of adaptation began they became less parasitic and more commensal in their relationship, until finally some have become definitely helpful to their hosts in a mutualistic relationship. It is probable that the regular immunity processes of insects had something to do with the insect's ability to overcome the destructive effects of the originally parasitic organisms. Evidence that such a process is still going on may be found among the rickettsiae: *Rickettsia prowazekii* de R.-L., the cause of human typhus, is to some extent pathogenic to its insect host, the louse, and probably is not so far along in its evolutionary process of intracellular adaptation as is *Rickettsia rickettsii* (Wolb.), the cause of Rocky Mountain spotted fever, which apparently causes its tick host no harm whatsoever.

If another line of speculation is followed it may be assumed that the intracellular symbiotes represent early nonpathogenic forms which were derived from microorganisms usually associated with normal insects and which, through the use of cellular and humoral processes of immunity, are incorporated into the tissues that evolved to make up the mycetomes and other symbiote-containing tissues. This has been thought by some, (*e.g.*, Paillot, 1933) to have taken place in the case of the symbiotes of aphids and other insects that have symbiote-filled mycetomes and also occasionally have peculiar freely occurring bacteria in their hemolymph.

The role that intracellular symbiotes play in the life processes of their hosts probably corresponds to the position they have attained in the evolutionary processes of adaptation between them and their hosts. It is conceivable that some of the intracellular forms have only recently ceased causing their hosts harm and live in the cells of their hosts only as commensals. Such symbiotes are of no distinct benefit to their hosts, but they do enjoy the protection and food essentials furnished them by their hosts. It is likely that most intracellular symbiotes are of some benefit to their hosts, and many of them may be absolutely essential to the life of the insect. It appears almost certain that in nature the vast

majority of the symbiotes are dependent upon their hosts since few, if any, are known to live freely in nature apart from their hosts.

The intimate association between the intracellular symbiotes and their hosts itself speaks for the mutualism of the relationship. In most cases, every individual of an insect species carries the symbiotes. Furthermore, the changes that occur in the invaded cells or in the mycetocytes are not pathological, in the ordinary sense of the word, and cause the host no injury. An indication of the close relationship between the symbiotes and their host is the elaborate and complicated manner in which many of them are transmitted from one generation of the host to the next. Not to be overlooked is the fact that although at certain periods the symbiotes multiply and increase rapidly in the tissues of their host, yet at the proper time the microorganisms are rigidly controlled numerically and are not permitted to increase to such a point that they might prove fatal.

In recent years experimental evidence has been accumulating which shows not only that most of the symbiotes are harmless to the tissues of their host but that, in addition, they are distinctly beneficial to them. Much of this experimental evidence is based on what happens to insects when their symbiotes are removed. This removal is brought about by several methods, including (1) direct removal by dissection or centrifugation, (2) removal by placing the insect in an unfavorable environment, (3) removal by preventing their transmission to the next generation, and (4) removal by the action of antibiotics and chemicals.

The first of these methods was used by Aschner (1932, 1934) and by Aschner and Ries (1933). These workers removed the symbiotes from the human louse, *Pediculus humanus* Linn., by dissecting out the mycetome and by centrifuging the eggs. The resulting symbiote-free insects had their powers of reproduction and nutrition greatly impaired, and they did not live so long as did normal insects. These harmful effects could be partly reduced by the rectal injection of yeast extract.

The second method of removing the symbiotes, *i.e.*, placing the insect in unfavorable surroundings, has been used by Koch (1936) in the case of larvae of the saw-toothed grain beetle, *Oryzaephilus surinamensis* (Linn.). If this insect is held in a temperature of 36°C. for a considerable length of time, its symbiotes undergo a gradual degeneration and finally disappear. Koch noticed that such insects showed no obvious ill effects, although this fact does not mean that the symbiotes are not useful—only, perhaps, that their type of usefulness has not been determined.

The third method, that of removing the symbiotes by preventing their transmission to the next generation, has been used with the drugstore beetle, *Stegobium paniceum* (Linn.), and the cigarette beetle, *Lasioderma serricorne* (Fabr.). Fraenkel and Blewett (1943) removed the yeastlike

symbiotes from the external surface of the eggs of these insects, thus preventing the newly hatched larvae from acquiring the microorganisms as they ate their way through the chorion. The symbiote-free insects failed to grow normally and were unable to complete their development unless vitamins of the B group were supplied in their diet. Yeast was found to supply the necessary vitamin factors, and it is presumed that in normal insects the symbiotes provide the vitamin requirements.

One of the most interesting methods of removing the symbiotes is that of using such substances as penicillin and the sulfa drugs. In 1945, Brues and Dunn administered penicillin to cockroaches (*Blaberus cranifer* Burm.) and found the symbiotes to become greatly reduced in numbers or to be entirely destroyed. The symbiote-free insects died in several days, thus indicating their dependence on the intracellular microorganisms. A year later, Glaser (1946) reported that the symbiotes in *Periplaneta americana* (Linn.) males and females can be adversely affected or destroyed by sulfathiazole, sodium and calcium penicillin, or by maintaining the insects for a prolonged period at 39°C. These treatments prevented the development or caused the regression of the female sex glands, though the male glands were not affected. Glaser concludes, therefore, that the bacteria are in symbiotic relationship with their female hosts and are closely connected biologically with the development of the female sex glands. In contrast to Brues and Dunn, however, Glaser attributed the death of those roaches which succumbed not to the absence of symbiotes but to the direct toxic action of the drugs. Given proper diet, roaches without their intracellular bacteria did not appear to suffer in health, except as noted above.

One of the most significant advances in ascertaining the role of intracellular symbiotes in the life economy of their host has been the discovery that, in some insects at least, these microorganisms are capable of fixing atmospheric nitrogen in a manner making it available to the host insect. This has been most clearly indicated with the symbiotes of aphids, although similar data have been obtained with the symbiotic microorganisms of certain other insects (Tóth, Wolsky, and Bátori, 1942; Goetsch, 1946; and Tóth, 1946). It is assumed (Tóth, 1943) that the amino acids made available to the insect host are built up in much the same way as they are in the case of root-nodule bacteria of leguminous plants. Indeed the similarity is so striking that the bacteria have been included in the same genus, *Azotobacter* (Peklo, 1912, 1946). Tóth's work, however, has been challenged by Smith (1948) who has been unable to obtain results comparable to those of Tóth's, and who, therefore, maintains that the symbiotes of the insects studied do not fix nitrogen. It appears that further investigation is necessary to clear up this point.

From the foregoing statements, it may be concluded that although in many instances the life of the insect host is not necessarily dependent upon the intracellular symbiotes it harbors, nevertheless many insects do require their presence. In some cases at least, the symbiotes apparently furnish their hosts with essential substances, such as vitamins, which are lacking in their regular diet; or they supply hormones which aid in the development of the ovaries. It appears quite possible that the symbiotes of many insects are capable of fixing atmospheric nitrogen. Since it seems that the symbiotes cannot live in nature apart from their hosts, we may assume that they too derive benefit from this entomic relationship. Thus, in the majority of instances, the relationship between the insect and its intracellular inhabitants is one of mutual benefit, sometimes not always clear as to its nature, and may be considered a distinct case of mutualism.

The Mycetome and Its Function. A great variety of cells in the body of an insect may normally contain intracellular microorganisms, and frequently these cells are in no sense specialized. In a large number of insect species, however, a special structure, or "organ," is present, the principal function of which appears to be that of housing the symbiotes. This structure has been called by several different names; but when it was determined that it harbored microorganisms, supposedly of a fungous nature, it was called (by Šulc, 1910*a*,*b*) a "mycetome." The individual cells that make up the mycetome are known as "mycetocytes" or sometimes, when the symbiote is a bacterium, "bacteriocytes." (Mycetomes consisting of bacteriocytes are occasionally referred to as "bacteriotomes.") A mycetocyte does not necessarily have to be enclosed within a mycetome. Any cell that contains symbiotes may properly be called a "mycetocyte."

Not all insects have a mycetome, but in those that do the organ is usually located in the abdomen. In some insects it is very small; in others it may occupy a large part of the abdominal cavity. The size of the mycetome often varies with the age or stage of the insect, as well as with its sex, usually being larger in the female than in the male. In some species the mycetome is a single small body; in others it occurs in the form of pairs or even as a group of small mycetomes. It is highly colored and relatively easy to distinguish in some insects, while in others it is white, colorless, or transparent and very difficult to distinguish from the surrounding tissues.

The mycetome is as much a part of the insect possessing it as is any other tissue of the body. Its embryological formation has been well studied in several insect species, the aphid being a good example. Uichanco (1924) and others have shown that the follicular epithelium of the adult ovariole is invaded by the symbiotes, which later penetrate the yolk of

the developing eggs. Within the posterior portion of the egg, the symbiotes multiply rapidly as the vitellophages or "mycetoblasts" move toward them and together form a syncitial mass. The mycetoblasts undergo mitotic division, and in a very precise manner the mycetome is formed. At first it consists of a single group of mycetocytes, then it divides into two lateral halves, and finally it becomes the heavily tracheated, longitudinally bipartite organ of the adult aphid. Within the mycetome the symbiote-containing mycetocytes number 60 or 70 large (approximately

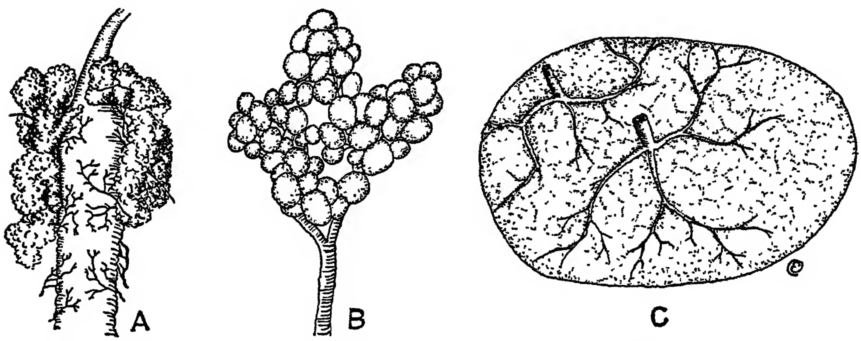


Fig. 38. Three of the various types of mycetomes found in insects. *A*. Mycetome attached to intestine of a weevil larva (*Lixus*). *B*. Mycetome from larva of *Cicada orni* Linn. *C*. Mycetome from *Pseudococcus maritimus* (Ehr.). Remnants of tracheols are indicated in each drawing. (*A redrawn from Buchner, 1939; B redrawn from Šulc, 1910; C original.*)

95 microns in diameter) cells with prominent nuclei. The symbiotes occupy the cytoplasm of the mycetocytes and never invade the nuclei. As the aphid goes through the various activities of its adult life, the mycetocytes one by one degenerate, and by the time the aphid dies very few of the cells remain.

Early observers thought of the mycetome as having a number of functions; accordingly they gave it a variety of names. Because it resembled the vitellus or "yolk" of an impregnated ovum, Huxley (1858) called it the "pseudovitellus." Metchnikoff (1866*a*,*b*) called it a "secondary yolk," attributing to it a nutritive function; but since it became larger during the embryo's development instead of smaller as a yolk would do, this idea was dropped. Among the first insects observed to have mycetomes were aphids. Balbiani (1866) believed that their mycetomes were somehow connected with the sex of these insects and that an elucidation of their function would explain parthenogenesis. Some thought that the mycetome had a nutritive function, others that it had an excretory function, and still others that it was analogous to a plant gall produced in response to some stimulation or irritation, and so it went—almost every worker having

his own theory as to the purpose of this organ. Only after it was discovered that the cells of this structure were filled with microorganisms was the emphasis shifted from the function of the mycetome as an organ to the symbiotes that it contained. It is now generally believed that the mycetome itself has no function of its own directly affecting the life processes

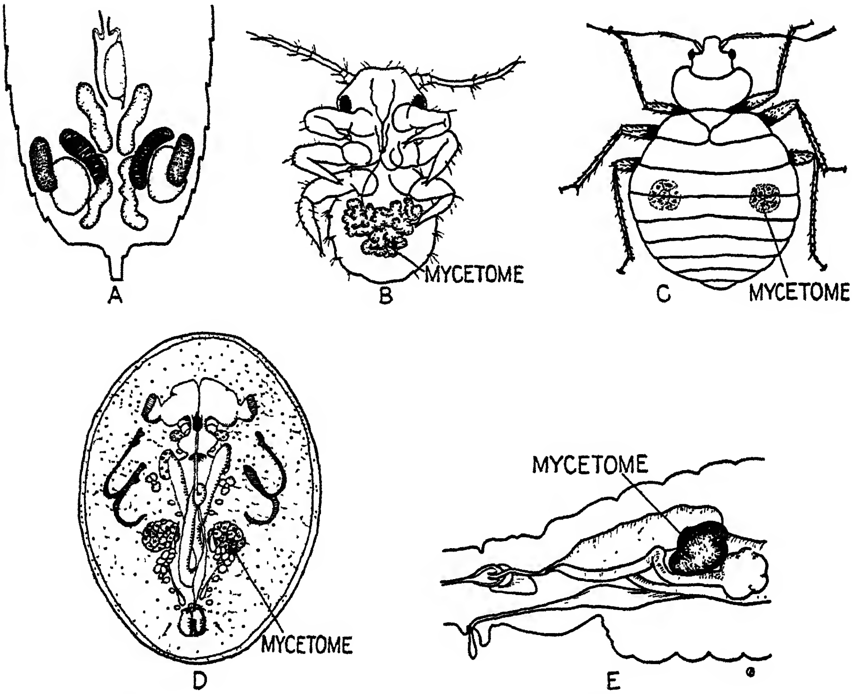


Fig. 39. Location of mycetomes in certain insects. *A*. The four different symbiote-harboring mycetomes in female Cixiinae. *B*. Nymph of *Psylla buxi* (Linn.), showing mycetome in the abdomen. *C*. The bedbug, *Cimex lectularis* Linn., showing the location of the paired mycetomes. *D*. *Trialeurodes vaporariorum* Westwood, showing the location of the paired mycetomes. *E*. Lateral view, showing one of the mycetomes of *T. vaporariorum* Westwood. (*After Müller, 1940; Buchner, 1939; and Weber, 1930.*)

of the insect. Instead its principal function appears to be simply that of sheltering and housing the symbiotes.

Types and Arrangements of Symbiotic Tissues. The tissues that contain the intracellular symbiotes are of many types and are arranged in an exceedingly great number of ways within the insect body. It is not practical here to review all the various types and arrangements that do exist, but it is important for the student to keep in mind the principal ones, and these we shall mention briefly. Certain of them will be considered again later in this chapter.

Pertinent to our subject are those extracellular symbiotes which live constantly in the lumen of the gut and its various pouches, caeca, and diverticula. This type of mutual relationship may be considered as a primitive type and possibly a forerunner of the intracellular association with which we are concerned in this chapter. The next step quite naturally

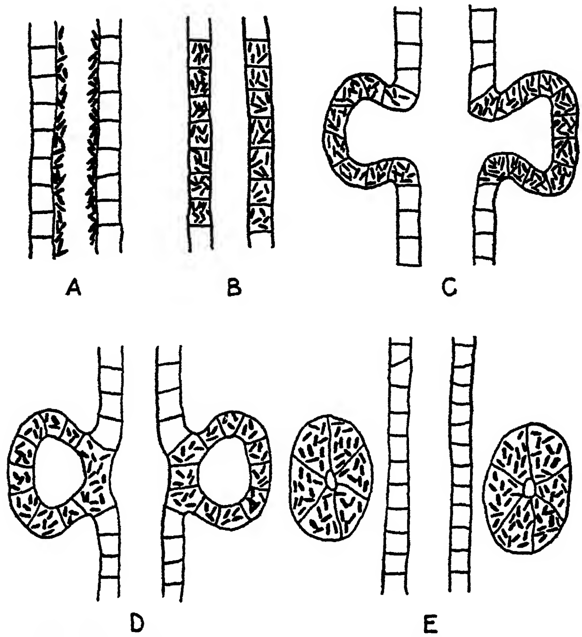


Fig. 40. A very diagrammatic representation of the principal anatomical locations of symbiotes with regard to the alimentary tract of the insect. Not all types or variations are shown. *A*. Symbiotes extracellular but lying rather close to the intestinal epithelium. *B*. Symbiotes situated within the normal epithelial cells. *C*. Symbiotes in epithelial cells of the intestinal wall which forms diverticula or outpocketings at this point. *D*. Outpocketing closes off forming mycetomes still attached to the gut. *E*. Symbiotes situated in mycetomes completely separated from, but lying close to, the gut.

would appear to be the invasion of the epithelial cells lining the gut. Usually it is the cells of the midgut that are involved, such as is the case with *Rhodnius prolixus* Stål, in which epithelial cells contain bacteriumlike symbiotes. Numerous other examples of this, the simplest type of intracellular symbiosis, exist in many insects. In certain beetles we have a further development: the epithelial cells have become modified, sometimes to the extent of forming an actual outpocketing of the cells to form a structure similar to a mycetome (and indeed it is considered as such).

The next type of symbiotic arrangement is somewhat more complicated.

We refer to those instances in which the symbiotes occur in special mycetocytes located in the fat tissues of the insect. This arrangement exists in nearly all cockroaches and has been fairly well studied in these insects, as well as in various Homoptera. In different species of insects the mycetocytes may be situated in different locations in the fat body. These symbiote-containing cells may occur scattered singly or in small groups throughout the fat tissue, they may occur in parallel rows, they may occur in the center or toward the periphery, etc. Instead of the fat body, other tissues may include mycetocytes of diverse types. Indeed there are probably a multitude of instances, yet unknown, in which the symbiotes are contained in scattered or loosely grouped mycetocytes in various tissues and in various locations in the body.

We come now to the true mycetomes, which may be relatively simple in form and arrangement or extremely complicated. Nevertheless all are discrete organized cell complexes, usually surrounded by an epithelium. We shall not attempt to discuss the great variety of types known to exist. We may, however, mention four large groups of types. Variations of these four types may be found in several of the order of Hexapoda, but they have been particularly noted in the Homoptera. The four general types according to Beier (1938*a*,*b*) are: (1) Singly formed mycetomes harboring only one symbiote. Up to five different organs of this type may exist in a single insect. (2) Mycetomes consisting of two rather loosely joined organs not enclosed in a common epithelium. This type usually harbors two different symbiotes. (3) Mycetomes consisting of two zones but surrounded by a common epithelium and therefore joined into a single organ and containing two distinct kinds of symbiotes. (4) Mycetomes enclosed in a common epithelium and harboring three different kinds of symbiotes. Under each of these general types may be grouped a large number of variations and pseudotypes, giving insects an almost unlimited number of possible symbiotic arrangements.

It may be assumed, with reasonable safety, that the longer a symbiote has lived in the body of an insect species the more intimate becomes the association and the more involved becomes the symbiotic arrangement. The degree of complexity relating to the histological character of the tissue that harbors the symbiotes, the manner of transmission, and the embryological development all indicate the tenure of the relationship. Symbiotes that are acquired recently are, in general, less closely associated with the insect. Morphologically such symbiotes are more like their original free-living predecessor.

In addition to the different arrangements assumed by the symbiote-containing cells or mycetocytes, manifold types or methods of symbiote

transmission from generation to generation also occur. A detailed account of these methods is not possible here, and the reader's attention can be called to them only in a brief way. As has already been indicated in the case of aphids, the transfer of symbiotes from parent to offspring may be an extremely complex procedure involving the fundamental embryological development of the insect. The manner in which the symbiotes gain access to the developing eggs may vary from a direct penetration into

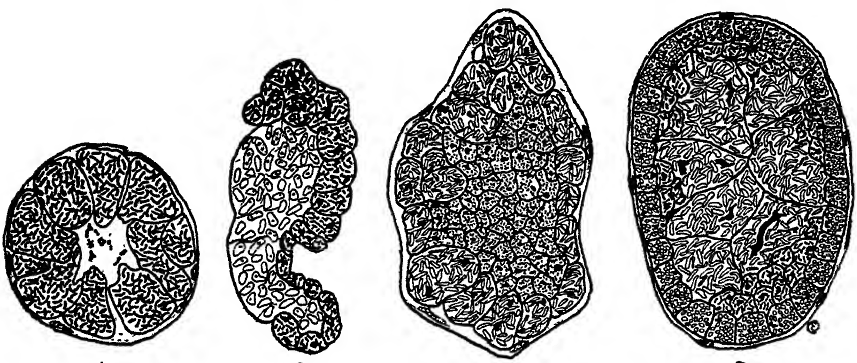


Fig. 41. Diagrammatic representation of the principal types of mycetome-symbiote arrangement in Homoptera, as described by Beier (1938). *A*. Singly formed mycetome harboring one kind of symbiote. *B*. Mycetome consisting of two more or less loosely joined tissues, not enclosed in a common epithelium, and harboring two different kinds of symbiotes. *C*. Mycetome consisting of two types of tissue surrounded by a common epithelium and therefore joined into a single organ. Harbors two distinct types of symbiotes. *D*. Mycetome having three different types of cells (mycetocytes) enclosed by a common epithelium, and harboring three different kinds of symbiotes.

it from the follicular lining or by way of the nurse cells, to entrance via the micropyle of the egg. In some beetles the latter method occurs when the symbiotes pass with the sperm during copulation into the bursa copulatrix of the female, and thence through the micropyle of the egg during its passage to the outside. In other cases the symbiotes are smeared on the exterior surface of the egg, or are buried within the chorion, and are acquired by the insect as it eats its way out of the egg. In nearly all instances, however, the passage of the symbiotes appears to be a passive one. Nevertheless, the transfer is always accomplished, and whatever the method it merits fascinated wonder.

Phylogenetic Relationships. Some study has been made concerning the relation of the various symbiotic types to the systematic position of their insect hosts. Large groups have been very inadequately studied from this standpoint: but, in those which have received a thorough ex-

amination some interesting phylogenetic relationships have been noted. Buchner (1940) has discussed certain aspects of this subject in a review from which we cite a few examples.

Throughout some of the existing systematic groups of insects the symbiotic picture is quite unified even up through the suborders. All the aleyrodids, for instance, have the same type of symbiosis and the same manner of symbiote transmission, in which a number of intact mycetocytes are carried over in the ovum. A similar uniformity exists in the psyllids. In the superfamily Aphidoidea, on the other hand, similarity of type is limited to families. Thus Aphidae and Eriosomatidae (= Pemphigidae) have rounded symbiotes, Adelgidae (= Chermesidae) have rod-shaped symbiotes, and Phylloxeridae are apparently free of intracellular microorganisms. In the coccids there is no uniformity of type except in subfamilies. All the Lecaniinae have similar yeastlike symbiotes in the hemolymph and in the fat cells; the ortheziids contain bacteria in the fat bodies; the diaspids harbor degenerate rounded bacteriods. The mode of generation-to-generation transmission in these cases is also specific for the subfamily. Among the monophlebines all the genera have paired elongate mycetomes, although *Marchalina* appears to be an exception. In this genus the symbiotes are carried in greatly enlarged cells in the gut epithelium. This discrepancy is clarified if one accepts the rearrangement presented by Morrison in 1928, which removes *Marchalina* from the monophlebines and places it as a tribe in the new subfamily Coelostomidiinae. One wonders if many similar changes would not be made if the taxonomist had the advantage of knowing the symbiotic arrangement of the insects with which he worked.

In the scale insects at least it appears that, by and large, the subfamilies arose before the symbiotes were acquired. This is indicated, for example, in the Tachardinae, where there is no uniformity of symbiotic types until one is within the confines of the tribes. Above this category one is likely to find one group with yeastlike symbiotes in the fat tissues, and in another group mycetocytes containing other types of symbiotes. The manner of transmission is also found to vary in these groups. A similar situation prevails with the suborder Cicadoidea, which must have developed into its present form before it entered into its numerous symbiotic alliances. The subfamilies are very easy to distinguish by virtue of their symbiotic arrangements. Thus the gaeanines contain a yeast in the fat tissue and a bacteriumlike symbiote in the mycetome; cicadines contain no yeast but have two different rod-shaped organisms. Similarly, the hassids have their particular type of organization, and most of the other present taxonomic categories have a similar uniformity in this respect. There are, however, interesting discrepancies. For example, the genera *Euacanthus*

and *Tettigoniella* are usually placed with the proconiines, although symbiotically they differ markedly from each other and according to some systematists should actually be placed in two separate tribes. Such is the case among the cicadas. In the superfamily Fulgoroidea, 186 species have been investigated with regard to their symbiotic types, and there appears to be even less phylogenetic agreement between subfamilies and tribes than in the Cicadoidea. In this connection the student is referred to the excellent monograph on the symbiotes of fulgorids by Müller (1940). (See also the paper on the symbiotes of Membracidae by Rau, 1943.)

It is worth drawing attention to the fact that the food habits of many insects are correlated with their systematic position and that great differences in this regard occur at the family level. Since it is thought that many symbiotes play a nutritive role in some insects, the correlation here may be more than meets the eye.

Some workers have been able to use the characteristics of the symbiotes to differentiate even species of insects. Mahdihassan, in India, asserts that he has been able indirectly to differentiate species of coccids by examining bloods smears containing their symbiotic yeastlike organisms, these symbiotes showing morphologically distinct forms dependent upon the species of insect harboring them.

Types of Intracellular Symbiotes. The wide ranges of variation among the various types of symbiotic tissues have already been discussed. Similar variations occur among the symbiotes themselves. Morphologically the symbiotes may vary with the group of insect as well as with the type of symbiotic tissue. As was brought out in the preceding paragraphs, within insect groups as large as tribes, subfamilies, or even families, there may be a uniformity not only of the type of mycetome but of the kind of symbiote as well. There may, however, be variation within the lower taxonomic categories.

Some of the morphological variation of symbiotes may be related to the length of time they have been associated with their host. According to Buchner (1940), recently acquired symbiotes are less intimately associated with the insect and are morphologically more similar to the original free-living microorganisms. As additional symbiotes enter the animal, each successive one shows less modification from its original form. Thus it is possible to tell which was the original symbiote. In aphids, for example, the common rounded symbiote is the original, or principal, one. To these have been added coccoid, rod-shaped, threadlike, and other forms.

For convenience the intracellular symbiotes may be broadly separated into three large morphological groups or forms of microbial life: bacteria, rickettsiae, and yeasts. Included within each of these groups are micro-

organisms that may be designated as bacteriumlike, rickettsialike, and yeastlike. The remainder of this chapter will be devoted to a brief consideration of a few examples of each of these groups.

To these three large groups those protozoa which live a large part of their life within the cells of insects might be added. Since in nearly all cases these protozoa are distinctly parasitic in nature we shall reserve our consideration of them until we discuss protozoan infections in insects.

INTRACELLULAR BACTERIAL AND BACTERIUMLIKE SYMBIOTES

It is not always possible to distinguish the bacterial symbiotes from those grouped as rickettsiae or as yeasts. Rickettsiae are probably only a peculiar type of bacteria, and the question of whether certain forms are yeasts or bacteria probably will not be settled until they have been cultivated and studied apart from their hosts. Accordingly, any present grouping is subject to change depending upon a more complete study of its members.

Symbiotes of Cockroaches. Among the first to be discovered and the best known of the intracellular bacterial symbiotes of insects are those found in cockroaches. Apparently all species of blattids carry them—principally in certain cells (bacteriocytes) in the fat body. They are also found in the egg, developing embryo, follicular epithelium, and the peritoneal sheath of the ovaries and the testes.

In a living condition the symbiotes are gram-positive, and they may stain uniformly homogeneous or banded, often with a clear central area and a bipolar arrangement of chromatic material. These and other morphological characters appear to vary depending on the species of roach, the stage of the development of the roach, the physical condition of the roach, the location in the roach (*i.e.*, egg or fat body), and similar factors. Cultivation outside the host has been claimed by several investigators; others have been unable to obtain positive results (see Gier, 1946; Gubler, 1947). Some of these claimants were undoubtedly working with contaminants; others, like Glaser (1930*a*,*b*; 1946), maintain the accuracy of their results despite the fact that some workers have been unable to offer confirmation. The bacteria isolated by Glaser were diphtheroids to which he gave the names *Corynebacterium periplanetae* var. *americana* [from the American cockroach, *Periplaneta americana* (Linn.)] and *Corynebacterium blattellae* [from the German cockroach, *Blattella germanica* (Linn.)]

In the fat body the symbiote-filled bacteriocytes are arranged in different ways in different species. They may occur simply scattered at random throughout the fat tissue, or they may lie longitudinally as a row or rows in the center of the fat body. The number of rows of bacteriocytes

varies from 1 to 20 with always at least 1 row of fat cells between. Rarely are the bacteriocytes found in the peripheral fat lobes.

Transmission of the symbiotes from parent to offspring takes place by way of the egg. The oöcytes in the ovaries are surrounded by one or more layers of symbiotes which penetrate through the oöcyte membrane into the cytoplasm. Thus within the developing embryo they move in masses to the center of the yolk. As the insect is formed, some of the organisms from this mass are carried into the body cavity, where they are taken up by the cells of the lateral lobes of the fat bodies while a few are caught between the cells of the developing ovaries. Here they lie dormant within the ovarioles until, as the time for egg laying approaches, they multiply rapidly and spread over the surfaces of the enlarging oöcytes. The entire process is then repeated.

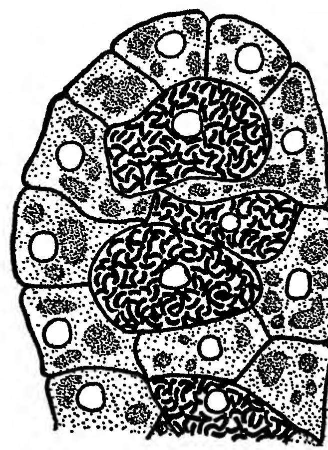


Fig. 42. Section of a lobe of the fat body from *Blatta orientalis* Linn., showing symbiote-containing mycetocytes. (*Redrawn from Gier, 1936.*)

As has been mentioned elsewhere, there are indications that the function of the symbiotes has to do with the development of the female sex glands, perhaps supplying these with some constituent lacking in the diet. At any rate, the symbiotes appear to be in a mutualistic relationship with their hosts.

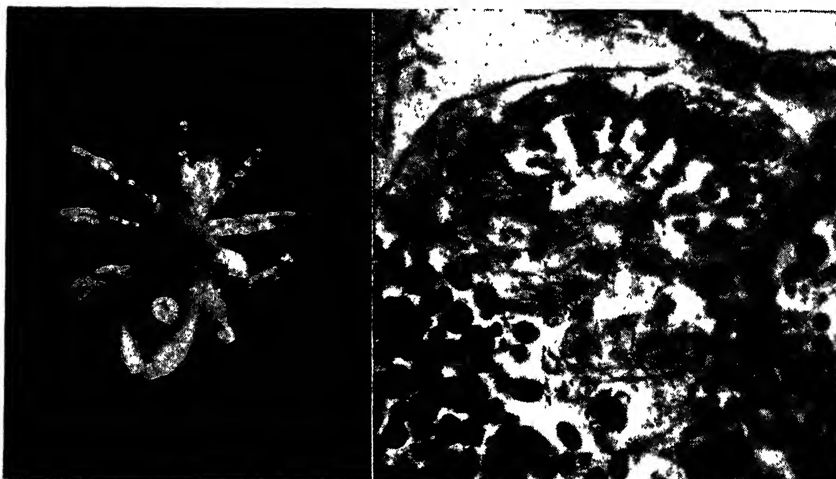
Symbiotes of Lice. Lice, particularly the species infesting human beings (*Pediculus humanus* Linn.), have for years been known to be one of the principal insect carriers of disease organisms, including the spirochete of relapsing fever and the rickettsia of typhus. In addition to these organisms pathogenic for man, lice harbor symbiotes harmless to themselves as well as to man. Indeed, as was pointed out in an earlier paragraph, these symbiotes appear to be necessary for the maintenance of the louse's existence. Without them the insect's nutrition and powers of reproduction are greatly impaired.

The symbiotes are long, rod-shaped, bacteriumlike organisms occurring in groups and, in the body louse, enclosed in a mycetome known as the "stomach disc." This small structure is spherical or oval in shape and is attached to the outer ventral midgut, where it lies exactly in the midventral line of the body, slightly nearer the anal region than the head. It consists of cells derived from the midgut and is covered with an outer layer of mesodermal tissue. The symbiotes of the human body louse are

enclosed in the 12 or 16 chambers into which the mycetome is radially divided. In the pubic louse (*Phthirius pubis* (Linn.))., the mycetome consists of 20 to 24 chambers.

The symbiotes are transmitted to the next generation of lice when they invade the ovaries and gain entrance to the egg through the egg stem.

Other species of lice also harbor symbiotes. The hog louse, *Haematopinus suis* (Linn.), harbors large, pleomorphic, homogeneous rods in my-



A B

Fig. 43. Symbiosis in the body louse, *Pediculus humanus corporis* DeG. *A*. Ventral view of an immature louse showing the position and shape of the mycetome (stomach disc, or *Magenscheibe* of German authors). *B*. Sagittal section through the stomach disc of a louse embryo showing the chambers within which lie the symbiotes. (*From Aschner, 1934.*)

cetomes located on the wall of the midintestine. All attempts to cultivate them have been unsuccessful. Transmission is transovarial. Similar symbiotes occur in cattle and horse lice. Other symbiotic arrangements are present in the dog louse and in the rat louse, which have azygous mycetomes.

Symbiotes of Aphids. As a group, the aphids, or plant lice, are as intimately associated with their intracellular symbiotes as are any insects. We have already mentioned the complex and yet regular embryological development of the aphid mycetome and the transmission of the symbiotes through the eggs of the insect. The nature of the symbiotes themselves, however, has had less elucidation, and complete agreement does not exist as to whether they represent bacteria or yeasts. In recent years increased evidence has accumulated to the effect that they are really of

the nature of bacteria, and we shall consider them as such for our purposes here.

A variety of morphological forms may represent the symbiotes in different species of aphids. Sometimes the same symbiote appears in several shapes and sizes in an individual insect. Thus large round or spherical forms may occur along with filamentous types or small bipolar bacilli. It has been presumed that all the forms in one insect arise from one simple form. When the symbiotes of aphids are examined, it should be remembered that these microorganisms are extremely pleomorphic and that almost any bizarre type may be expected. In most cases, however, the typically small rod-shaped bacteria and the larger round forms are present. The rod-shaped symbiotes, upon staining, are frequently barred or vacuolated. The round ones may have slightly irregular contours, are not vacuolated, and have a fairly homogeneous protoplasm. Attempts to cultivate the symbiotes of aphids have been reported successful in a few instances, but most efforts have failed.

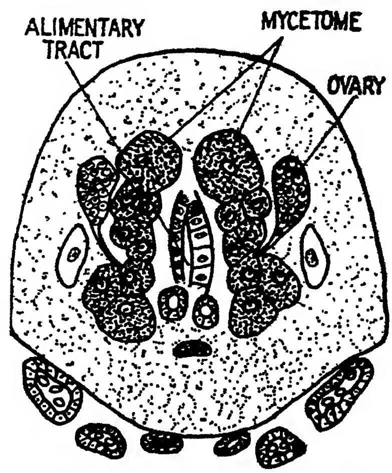


Fig. 44. Cross section through the subcaudal portion of the abdomen of the aphid *Macrosiphum rosae* Linn., showing location of the mycetomes with respect to the ovaries and alimentary tract. (*Redrawn from Uichanco, 1924.*)

In most aphids the mycetome is a longitudinally bipartite organ consisting of a large number of mycetocytes, which decrease in number as the insect grows older, until at the time of its death only a few mycetocytes remain. The mycetomes of some aphids apparently contain two or three species of microorganisms, although it is frequently difficult to determine whether one is concerned with different morphological types of the same species. Some authorities believe the spherical-shaped organisms are the oldest and hence consider them the primary forms. The rod-shaped, or secondary, symbiotes are usually not so well localized in the mycetome or other symbiotic tissue and may occur free in the body cavity, blood cells, connective tissue cells, or elsewhere. The whole subject is one worthy of further study; and the true nature, characteristics, and associations of the symbiotes of aphids must await this study for further clarification.

It might be mentioned here that the adelgids have symbiotes in many respects similar to those of the aphids, but the mycetomes are different.

Most adelgids have mycetomes consisting of two strands or elongated groups of cells situated alongside the gut. In the Chermidae, or jumping plant lice, the mycetome in the young insect is a single structure, and in the adult it is a paired organ. It consists of a central syncytium surrounded by mycetocytes. The symbiotes may occur in the syncytium as well as in the mycetocytes but do not always do so. The symbiotes are usually elongated organisms rather than spherical, and they vary in size from short rods to long filaments.

Symbiotes of Mealybugs. There has been some difference of opinion as to whether the symbiotes of mealybugs are bacterial or yeastlike in nature. It is now generally conceded, however, that they are bacteriumlike symbiotes capable of marked pleomorphism.

In the genus *Pseudococcus* the symbiotes are found in a single, more or less oval, yellowish-orange, heavily tracheated structure lying below, or practically encircled by, the intestine and occupying about a fourth to a third of the length of the host's body. Each of the mycetocytes making up the mycetome has a large nucleus rich in chromatin. The rest of the cell is filled with spherical or oval colorless balls within which are located the symbiotic microorganisms. The apparent mucoid nature of these balls sometimes makes it difficult to demonstrate the microorganisms inside. In any case the symbiotes do not take stains so readily as do ordinary bacteria.

Some workers believe that the mycetome of certain mealybugs contains two different species of symbiotes, while others consider the microorganisms to be two forms of the same species. One form is usually a large, pleomorphic, yeastlike organism with a homogeneous protoplasm. The other is a smaller, rod-shaped, bacteriumlike organism. The form usually seen in the mycetome of the nymph or early adult may be described as a rather large, somewhat curved or bent, plump or sausage-shaped bacterium with blunt or rounded ends. At certain seasons of the year, usually spring, rounded or spherical forms may predominate, with elongated forms dominating during the winter. In certain species of mealybugs, *e.g.*, *Pseudococcus brevipes* (Ckll.), the small bacteriumlike form is thought to condition the insect's oral secretions in such a way as to cause them to produce green spots on leaves while feeding (Carter, 1936).

Transmission to the next generation occurs transovarially. The contents of the cell-like spheres of the mycetocytes may aggregate into small very dense and deeply staining bodies which migrate from the mycetome and enter the egg at its junction with the nurse cell. Or the symbiote-containing spheres or balls may themselves migrate to the egg and enter it. They are taken up in a depression at the anterior pole of the egg; from this position they are incorporated into the regular embryological development of the mycetome.

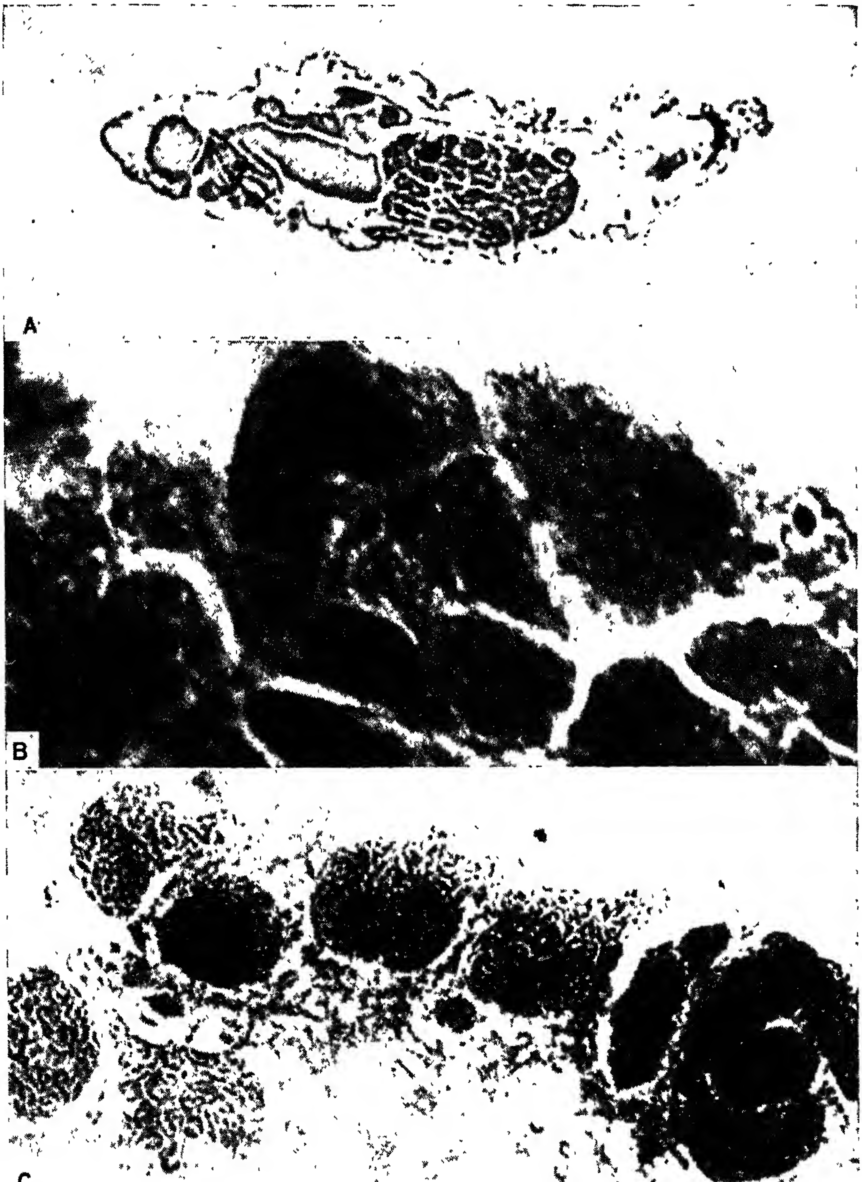


Fig. 45. Mycetome and symbiotes of the citrophilus mealybugs, *Pseudococcus gahani* Green. *A*. Longitudinal section of entire insect showing position of the mycetome (the large dark structure). *B*. A single mycetocyte with centrally located nuclear material and with the cytoplasm separated into several sections or parts. *C*. Groups of symbiotes from mycetome. (*Photographs by K. M. Hughes.*)

A great number of the intracellular symbiotes found in the Homoptera are yeasts or yeastlike organisms, and these will be taken into account in a subsequent section.

Symbiotes of Reduviidae (Assassin Bugs). Several species of blood-sucking reduviids have been found to harbor intracellular bacteria within the cells of the intestinal epithelium. *Rhodnius prolixus* Stål has been studied fairly well in this connection, and the symbiotes of certain of the *Triatoma* have had cursory examinations.

In newly hatched *Rhodnius* the cells lining the anterior narrow segment of the midgut are filled with bacteria. By means of vesicular swellings

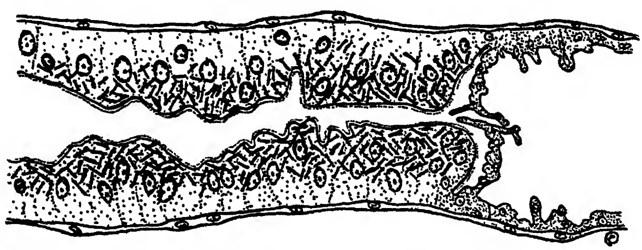


Fig. 46. Longitudinal section of the junction between the foregut and midgut of a nymphal *Rhodnius prolixus* Stål., showing location of symbiotes in the epithelial cells. (*Redrawn from Wigglesworth, 1936.*)

that extrude through the cell walls, these organisms are set free into the lumen of the gut when the insect molts. Here they multiply in the undigested blood meal until they themselves are apparently digested. Although at first believed to have the characteristics of a diphtheroid bacillus, the symbiote was later reported to be an actinomycete, *Nocardia rhodnii* (Erik.) (= *Actinomyces rhodnii* Erik.). In the absence of the microorganism, the insects grow and molt normally only until the fourth or fifth instar. From then on, molting is delayed or fails, and very few of the bugs become adults. If the insect is reinfected with the actinomycete, which may be grown on artificial media, all its normal activities are resumed. It is thought that the symbiotes supply necessary vitamin requirements—probably one of the B vitamins. Although at first it was thought that transmission took place through the egg, it is now believed that the young nymphs acquire the microorganisms from their environment such as through ingestion of the excreta of other members of the species or from the contaminated surfaces of the eggs (Wigglesworth, 1936; Brecher and Wigglesworth, 1944).

In *Triatoma rubrofasciata* (DeG.), Webb (1940) found symbiotes to occur intranuclearly as well as intracytoplasmically in the cells of the gut wall, salivary glands, ovaries, Malpighian tubes, and muscles. He

observed them in every stage of the insect, including the egg. Since this rickettsialike form is smaller in size than that in *Rhodnius*, of different distribution in the insect, present in the egg, stains gram-negative instead of gram-positive, is noncultivable on artificial media, and is somewhat pathogenic for laboratory animals, it appears that distinctly different types of symbiotes may occur in reduviids.

Among the other Hemiptera that harbor intracellular symbiotes, the bedbug, *Cimex lectularius* Linn., has at least one that it houses in a definite mycetome. Since this organism has been placed in the rickettsial group, we shall delay our consideration of it until we discuss this group in more detail.

Symbiotes of Beetles. A large number of Coleoptera are known to harbor intracellular symbiotes. In some cases (*e.g.*, in Anobiidae) the organisms are of the nature of yeasts; in other instances (Bostrichidae, Curculionidae, Lyctidae, and Cucujidae) they are bacteriumlike.

In the bostrichids, or powder-post beetles, the mycetomes are paired and located one on each side of the alimentary tract. The symbiotes are small pleomorphic bacteria which are transmitted to each succeeding generation of beetles through the agency of the male. The symbiotes are mixed with the sperm, which is deposited in the bursa copulatrix of the female. From here they pass through the micropyle of the fully formed egg while the latter is being oviposited.

Probably all Curculionidae (snout beetles) have bacteriumlike symbiotes associated with them. In the larvae the mycetomes are frequently nothing much more than a girdle of outpocketings from the walls of the alimentary tract, usually at the juncture of the foregut and the midgut. As the insect matures the mycetome becomes further developed, with definite mycetocytes containing the symbiotes. Various types of symbiotic arrangements, however, occur in different groups of snout beetles. A description of these types may be found in the writings of Buchner (1930, 1933).

Of the cucujids, or flat beetles, perhaps the best known example as concerns their symbiotes is that of the saw-toothed grain beetle, *Oryzaephilus surinamensis* (Linn.). The larva of this beetle has four mycetomes, two of which lie over the intestine in the first and second segments, while the other two are situated ventrally in the third and fourth segments. In the pupal and adult stages the first two mycetomes are drawn nearer together while the two posterior ones move farther apart. The mycetocytes have a central nucleus and a surrounding cytoplasm divided into cell-like regions that contain the microorganisms. These symbiotes are large vermiform organisms and are transmitted through the eggs by way of the follicles. It is of interest to note that Koch (1931, 1936) was able

to free the beetle of its symbiotes by holding the insects at a temperature of 36°C. The symbiote-free individuals apparently suffered no visible ill effects, and through 25 subsequent generations the descendants regularly developed sterile mycetomes.

Certain of the chrysomelids (*Cassida, Bromius, Donacia*) harbor symbiotes that may occur in both an intracellular and an extracellular location (Stammer, 1936). For example, they may occur intracellularly in certain intestinal caeca, and extracellularly in peculiar vaginal pouches. As the eggs are oviposited, the bacteriumlike symbiotes from the vaginal pouches are smeared over the chorion of each egg. When the larvae hatch, they ingest part of the eggshell, and thus the symbiotes have been passed to the next generation. The symbiotes are carried to small caeca at the beginning of the midgut as well as to the lumina of the Malpighian tubes.

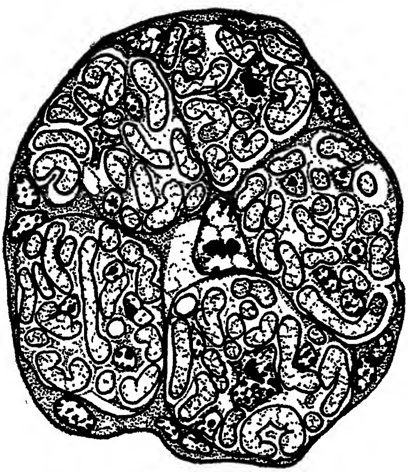


Fig. 47. Cross section of the mycetome from the larva of the saw-toothed grain beetle, *Oryzaephilus surinamensis* (Linn.), showing the symbiote-containing mycetocytes. (*Redrawn from Koch, 1936.*)

Symbiotes of Flies. The symbiotes of Diptera have not been so well studied as have those of Homoptera or Coleoptera. It is known, however, that different types of symbiotic arrangements exist among flies. In certain midges (*Dasyhelea*), for example, definite mycetomes, filled with bacteria, are present. In certain tabanid flies the pericardial cells and the Malpighian tubes contain small rods and filamentous microorganisms. Of the muscid flies, certain species of *Glossina* are known to harbor pleomorphic, gram-negative, bacteriumlike microorganisms in a ring of symbiotic tissue around the intestine.

The Pupipara, which live parasitically upon birds and mammals, have some symbiotes fairly well known for their similarity to rickettsiae. That of the sheep ked (*Melophagus ovinus* (Linn.)) has been placed in the genus *Rickettsia*. Others, the symbiotes of *Hippobosca, Nycteribia*, and most *Lipoptena*, have not been named, but in many respects they are similar to those of the sheep ked. Some of them occur extracellularly, while in many cases they inhabit certain tissues associated with the midgut or are located in masses of cells lying dorsally in the abdomen on either side

of the rectal sac. Several of the symbiotes have been cultivated on artificial media. In at least some of the insects the microorganisms are transmitted to the offspring through the milk glands of the females.

Symbiotes of Ants. Two genera of ants have been fairly well studied with regard to their intracellular microorganisms: *Camponotus* and *Formica.*

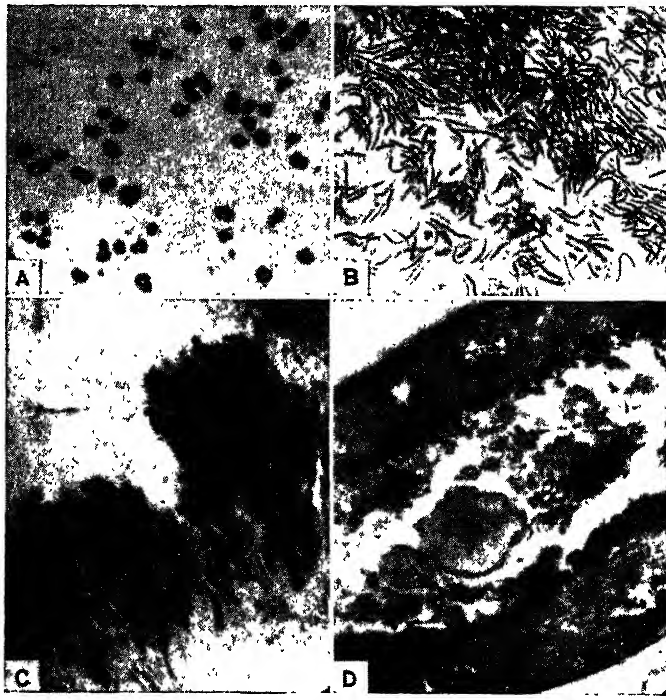


Fig. 48. Symbiotes of Pupipara. *A.* Symbiotes from *Hippobosca camelina* Leach. *B.* Symbiotes from *Ornithomyia avicularia* Linn. *C.* Section through the symbiote-containing cells of the intestinal epithelium of *H. equina* Linn. *D.* Section through the anterior part of the foregut of a larval *Hippobosca equina* Linn., showing the symbiotes attached perpendicular to the epithelium and lying free in the lumen. (*From Aschner, 1931.*)

In both genera the symbiotes are similar in morphology. In most instances they are gram-negative, usually slightly curved, bacteriumlike forms, varying in size from short thick rods to long slender organisms. In most species of ants the organisms are located in the epithelium of the midgut.

All castes of all species of the genus *Camponotus* appear to harbor symbiotes. Certain cells in the intestinal epithelium serve as mycetocytes within which the symbiotes may be seen to lie as bundles of long rods running parallel to each other and forming rings about the nucleus. In-

the genus *Formica* the mycetocytes are situated in a somewhat different fashion, lying in cells just behind the intestinal epithelium. In both genera transmission takes place via the egg (Lilienstern, 1932).

White ants, or termites, belong to the order Isoptera, but it may be mentioned here that these insects also have intracellular symbiotes, which have received some study (Koch, 1938*a*,*b*; Tóth, 1946).

Symbiotes of Ticks and Mites. In addition to Hexapoda, other arthropods have been found to contain intracellular microorganisms. Among the Acarina, for example, the ticks and mites have been examined from this standpoint (Cowdry, 1923; Buchner, 1926, 1930; Mudrow, 1932).

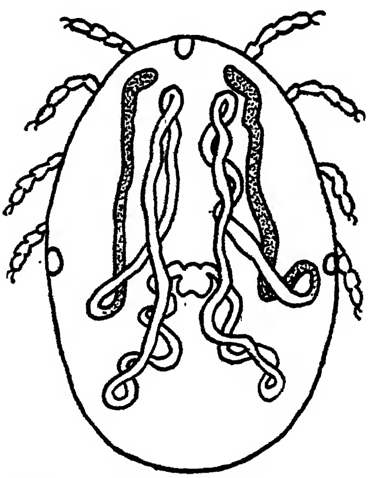


Fig. 49. A schematic representation of a female tick (*Rhipicephalus*), showing the portion (stippled) of the Malpighian tubes that carries symbiotes. (*Redrawn from Mudrow, 1932.*)

Both scutate (Ixodidae) and non-scutate (Argasidae) ticks have micro-symbiotes. Many of the microorganisms are rickettsial in nature; others are similar to larger bacterial forms. In most species of ticks only certain cells of the Malpighian tubes and ovaries are inhabited. Occasionally the cells of the diverticula and other tissues are involved. Transmission is transovarial, the symbiotes passing from the heavily invaded cells of the ovary to the developing oöcysts. The methods by which the microorganisms penetrate the eggs, however, differ between that in the Ixodidae and that in the Argasidae.

Unlike the ticks, certain of the mites have mycetomes (closely associated with their alimentary tracts) made up of symbiote-containing mycetocytes. As many as six different types of symbiotes have been reported in a single mite species, but each type is always located in an individual mycetocyte.

RICKETTSIAL SYMBIOTES

Were it not for the fact that certain of the intracellular symbiotes of insects and ticks cause diseases of man, the existence of the special group called "rickettsiae" might never have been recognized. Except for this pathogenic property of some of them, there are no essential differences between the rickettsiae and many of the rickettsialike organisms found in many widely separated species of insects. In fact, many of these non-

pathogenic forms have been given names and have been placed in the genus *Rickettsia* along with the forms pathogenic for man and other animals.

The generic name *Rickettsia* was established in 1916 by da Rocha-Lima to honor Howard T. Ricketts, an American investigator who died of typhus fever while studying its etiology in 1910. The word "rickettsia" is generally used as a common name for any one member of the group, much as the term "bacterium" is used to designate one of the bacteria. The plural form is either "rickettsiae" or "rickettsias." There are several genera of rickettsiae, the most prominent ones being *Rickettsia*, *Coxiella*, and *Cowdria*. Some authors place the rickettsiae of the spotted-fever group in the genus *Dermacentroxenus*. Several important points concerning the nomenclature and classification of these organisms are still in doubt.

The Nature and Characteristics of Rickettsiae. One reason why the rickettsiae needed a name of their own was that they could not logically be placed with the known bacteria, the viruses, or the Protozoa. Although they can be seen with an ordinary microscope, they cannot be grown on the types of artificial media used to cultivate most bacteria. In recent years, however, they are coming to be regarded as being closely related to the true bacteria and have been placed in the family Rickettsiaceae, order Rickettsiales. In any case they may be considered simply as a peculiar group of very small gram-negative bacteria living almost exclusively within or on the cells of their arthropod host, as well as intracellularly in the tissues of their vertebrate host when they have one. Their reaction and resistance to various chemical and physical agents are, in general, the same as those of most bacteria. The fact that most of them live and multiply in the bodies of insects or ticks is our reason for considering them here. Not only must the insect pathologist be able to recognize their presence in the tissues of the insects with which he works, but he should consider them in much the same light as he does other intracellular symbiotes. The fact that some of them cause disease in man is only of secondary biological interest—unless, of course, you happen to be the man.

The disease-producing forms probably acquired their first parasitism on arthropods, becoming so adapted to their intracellular existence that they could no longer exist as free-living bacteria. Most of them became so well adapted that they came to live in harmony with their hosts. As this evolution proceeded, some of the parasites were transmitted from the insects (especially those ectoparasites requiring blood meals) to some of the higher animals. These animals, and particularly man, are recent hosts, and one can only speculate on what the evolutionary future may hold for some of the other symbiotes associated with bloodsucking insects.

Not all arthropod hosts of rickettsiae are thoroughly adapted to invasion by the rickettsiae. The rickettsia that causes typhus also causes harm to its insect vector, the louse *Pediculus humanus* Linn. It would be difficult to better Zinsser's (1935) description of this relationship:

> The louse shares with us the misfortune of being prey to the typhus virus. If the lice can dread, the nightmare of their lives is the fear of some day inhabiting an infected rat or human being. For the host may survive; but the ill-starred louse that sticks his haustellum through an infected skin, and imbibes the loathsome virus with his nourishment, is doomed beyond succor. In eight days he sickens, in ten days he is *in extremis*, on the eleventh or twelfth his tiny body turns red with blood extravasated from his bowel, and he gives up his little ghost. Man is too prone to look upon all nature through egocentric eyes. To the louse, *we* are the dreaded emissaries of death. He leads a relatively harmless life—the result of centuries of adaptations; then, out of the blue, an epidemic occurs; his host sickens, and the only world he has ever known becomes pestilential and deadly; and if, as the result of circumstances not under his control, his stricken body is transferred to another host whom he, in turn, infects, he does so without guile, from the uncontrollable need for nourishment, with death already in his own entrails. If only for his fellowship with us in suffering, he should command a degree of sympathetic consideration.[1]

Although we have said that the ability of certain rickettsiae to cause disease in man is of only secondary importance to our biological consideration of them as intracellular symbiotes, it is nevertheless a convenient physiological property for separating them into two large groups: those which are pathogenic for vertebrates and those which are not. One reason for separating them is because the pathogenic forms have been described and studied much more thoroughly than have the nonpathogenic forms. Our consideration of both groups must be both brief and superficial. The interested reader may find more thorough treatments of the rickettsiae as organisms in accounts by Zinsser (1937), Steinhaus (1946), and others.

Pathogenic Rickettsiae. *Rickettsia prowazekii* da R.-L., the cause of typhus fever, is the most noteworthy of all the pathogenic rickettsiae. In the past it has been responsible for one of the world's greatest pestilences, nearly always associated with wars or periods of great unrest or movements among populations. It has frequently been instrumental in deciding the outcome of important military campaigns, and it has played a significant role in every major European war until World War II. Even in this war sporadic outbreaks occurred, but they were largely controlled through the use of vaccines and the application of insecticides such as DDT, which checked the vector, the human louse, *Pediculus humanus* Linn.

[1] Quoted with the permission of Little, Brown and Company, and the Atlantic Monthly Press.

The epidemic form of typhus caused by *Rickettsia prowazekii* is called "human typhus" to differentiate it from endemic, or murine, typhus, caused by its close relative *Rickettsia typhi* (W. & T.). Whereas *R. prowazekii* is transmitted by the louse from man to man, *R. typhi* is trans-

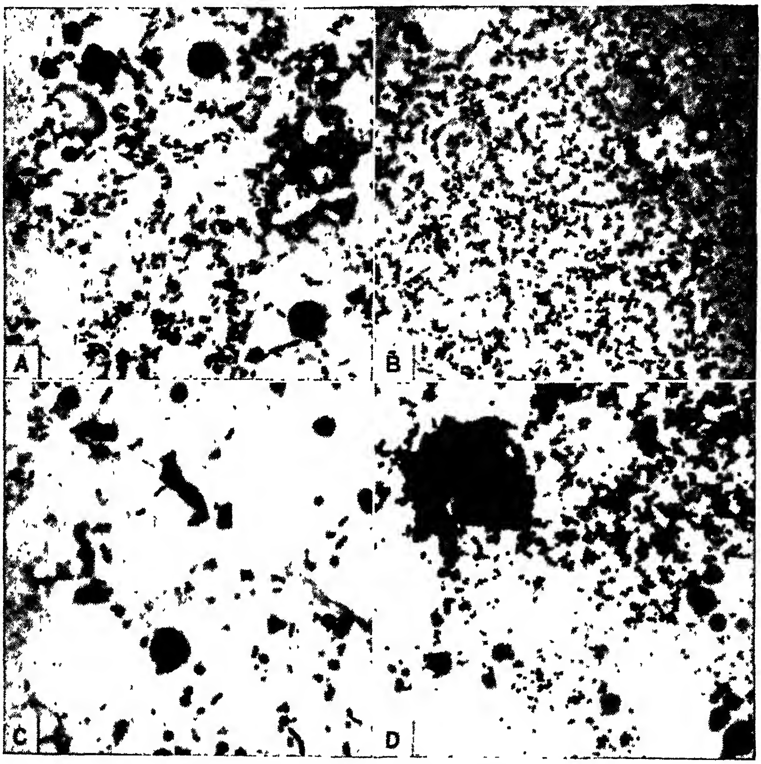


Fig. 50. Photomicrographs of well-known rickettsiae. *A. Rickettsia prowazekii* da Rocha-Lima, the cause of classical typhus in man. The small rod-shaped forms are the rickettsiae. *B. Rickettsia typhi* (Wolbach and Todd), the cause of murine typhus. *C. Rickettsia rickettsii* (Wolbach), the cause of Rocky Mountain spotted fever. *D. Coxiella burnetii* (Derrick), the cause of Q fever. (*From "Insect Microbiology," Comstock Publishing Company, Inc. Photographs by N. J. Kramis.*)

mitted from rat to rat by the rat flea, *Xenopsylla cheopis* (Roth.), or from rat to man by the same flea. Under certain conditions, the murine type may become epidemic in man by louse transmission from an infected to a healthy person, just as in the case of the classic human typhus. Transmission of either rickettsia by either the louse or the flea takes place through the contamination of the insect's bite with its highly infectious feces. We have already mentioned that *R. prowazekii* is somewhat patho-

genic for its vector the louse, but *R. typhi* shows no similar virulence for the flea.

Although tsutsugamushi disease, or scrub typhus, has been known to exist in Japan, Formosa, Sumatra, and other parts of the Far East for many years, it gained world-wide attention during World War II. Troops stationed in various islands throughout the southwest Pacific suffered losses due to this disease caused by *Rickettsia tsutsugamushi* (Hay.) and transmitted by larval mites, particularly *Trombicula akamushi* (Brumpt) and *T. deliensis* Walch. Trench fever, a rarely fatal disease of troops in the European theater during both world wars, is thought to be transmitted by lice in a manner similar to typhus. It is caused by *Rickettsia quintana* Schm. (or *R. wolhynica* J. & K.). Q fever (causative agent *Coxiella burnetii* (Derr.)) occurs in the United States and Europe (it occurred among troops in Italy during the last war), but it is probably best known as a disease among abattoir workers in Australia. *C. burnetii* is filterable through filters that retain most other rickettsiae. It may be transmitted by ticks, and in America it was first discovered in the tick *Dermacentor andersoni* Stiles.

Other than the agent of typhus, one of the best known rickettsiae is *Rickettsia* (*Dermacentroxenus*) *rickettsii* (Wolb.), the cause of Rocky Mountain spotted fever. This disease was discovered in the Rocky Mountain region of western United States where it is transmitted by *Dermacentor andersoni* Stiles. It is now known to occur in most other parts of the United States, as well as in distant parts of the world, several species of ticks being involved in its transmission. Unlike the rickettsia of typhus in the louse, the rickettsia of spotted fever is transmitted from one tick generation to the next through the egg. The rickettsia lives as a harmless symbiote in the cells of the intestinal diverticula and in other tissues of the tick. Its close relative, *Rickettsia* (*Dermacentroxenus*) *conorii* Brumpt, the cause of *fièvre boutonneuse* and South African tick-bite fever in Africa, is also transmitted by ticks.

Rickettsia akari H., J., & P. causes a disease in man known as "rickettsial pox" and is transmitted by mites (*Allodermanyssus sanguineus* (Hirst)).

Of the rickettsia type of organisms that are pathogenic for animals other than man, *Cowdria ruminantium* (Cowdry), *Rickettsia canis* D. & L., and *Colesiota conjunctivae* (Coles) are among the most prominent. The animals they infect and their vectors are indicated in Table 1 along with the other pathogenic rickettsiae.

None of the pathogenic rickettsiae live in distinct mycetomes in their arthropod hosts. Most of them are favored, however, by an intracellular habitat, and some, such as that of spotted fever, have become so well

TABLE 1. RICKETTSIAE PATHOGENIC FOR VERTEBRATES

Rickettsia *	Disease caused	Principal arthropod vectors
Pathogenic for man (and rodents)		
Rickettsia prowazekii da R.-L.	Human or epidemic typhus	*Pediculus humanus corporis* DeG. *Pediculus humanus capitis* DeG.
Rickettsia typhi (W. & T.)	Murine typhus	*Xenopsylla cheopis* (Roth.) *Nosopsyllus fasciatus* (Bd'A.)
Rickettsia quintana Schm. (= *wolhynica* J. & K.)	Trench fever	*Pediculus humanus corporis* DeG.
Rickettsia rickettsii (Wolb.)	Rocky Mountain spotted fever	*Dermacentor andersoni* Stiles *Dermacentor variabilis* (Say) *Amblyomma americanum* (Linn.)
Rickettsia conorii Brumpt	*Fièvre boutonneuse* South African tick-bite fever	*Rhipicephalus sanguineus* (Latr.) *Amblyomma hebraeum* Koch
Rickettsia tsutsugamushi (Hay.)	Tsutsugamushi disease, or scrub typhus	*Trombicula akamushi* (Brumpt) *Trombicula deliensis* Walch
Rickettsia akari H., J., & P.	Rickettsial pox	*Allodermanyssus sanguineus* (Hirst)
Rickettsia weigli Mosing	An unclassified rickettsiosis	*Pediculus humanus corporis* DeG.
Coxiella burnetii (Derr.)	Q fever	*Dermacentor andersoni* Stiles *Amblyomma americanum* (Linn.) *Ixodes holocyclus* Heum.
Several unnamed rickettsiae such as those causing:	Bullis fever; Maculatum disease (not recognized with certainty in man)	Bullis fever: *Amblyomma americanum* (Linn.) Maculatum disease: *Amblyomma maculatum* (Linn.)
Bartonella bacilliformis (Strong *et al.*) (Family Bartonellaceae)	Carrión's disease (Oroya fever)	*Phlebotomus verrucarum* Townsend
Pathogenic for animals other than man		
Cowdria ruminantium (Cowdry)	Heartwater	*Amblylomma hebraeum* Koch
Rickettsia suis D. & G.	Unnamed rickettsiosis of swine	Vector not reported

(Continued)

TABLE 1. RICKETTSIAE PATHOGENIC FOR VERTEBRATES (*Continued*)

Rickettsia *	Disease caused	Principal arthropod vectors
Rickettsia canis D. & L.	A rickettsiosis of dogs	*Rhipicephalus sanguineus* (Latr.)
Rickettsia bovis D. & L.	A rickettsiosis of cattle	*Hyalomma* sp.
Rickettsia ovina L. & D.	A rickettsiosis of sheep	Probably *Rhipicephalus bursa* C. & F.
Rickettsia avium Carp.	A rickettsiosis of birds	Unknown
Rickettsia pisces Moh.	A rickettsiosis of fish	Unknown
Colesiota (Rickettsia) conjunctivae (Coles)	A conjunctivitis of sheep	Vector undetermined. Mechanical transmission by flies suspected
Colesiota (Rickettsia) lestoquardi (D. & G.)	A conjunctivitis of swine	Vector not reported
Several unnamed species	Unnamed rickettsioses of bison and of guinea pigs (experimental)	Mostly unknown

* The systematics used are those of the sixth edition of "Bergey's Manual of Determinative Bacteriology" (Breed *et al*; 1948).

adapted to their arthropod host that they are even passed through the egg. So far no case is known in which a microorganism that inhabits a mycetome is pathogenic for man or other animals.

Nonpathogenic Rickettsiae. The tissue cells of many insects harbor small bacillary bodies morphologically indistinguishable from the well-known pathogenic rickettsiae we have just mentioned. As far as is known, the majority of them have no relation to diseases of man or other animals. A few have been given scientific names, and from a taxonomic standpoint they probably belong in the same general group with many of the pathogenic forms. There is no clear dividing line, however, between these named species and the many unclassified forms we have considered under the designation of "bacteriumlike" symbiotes. It is merely for temporary convenience then that we have separated them into an arbitrary category of their own.

At least one of the "nonpathogenic" rickettsiae lives in a mycetome—*Rickettsia lectularia* A., A., & B. This symbiote was discovered in 1921 by Arkwright, Atkin, and Bacot in the gut of the bedbug, *Cimex lectularius* Linn. About the same time, Buchner (1921, 1923) observed in the mycetome of this insect apparently the same microorganisms described by the British workers. Some authors (Pfeiffer, 1931) believe that both a

rickettsia and a true bacterium are present in the mycetome and other tissues of the bedbug, but this point has not been clarified. Each insect has paired mycetomes lying one on either side of the gut and near the gonads in about the third abdominal segment, and usually among the lobes of the fat body. Transmission of the organisms from one generation to the next apparently takes place via the eggs. The symbiotes themselves may appear as small cocci, or rods, or as longer threadlike organisms.

TABLE 2. "NONPATHOGENIC" RICKETTSIAE *

Rickettsia	Arthropod host	Principal tissue harboring rickettsia
Rickettsia melophagi Nöl.	*Melophagus ovinus* (Linn.)	Extracellular in midintestine; probably also intracellular
Rickettsia lectularia A., A., & B.	*Cimex lectularius* Linn.	Mycetome, intestinal tract, ovaries
Rickettsia rocha-limae Weigl	*Pediculus humanus* Linn.	Intracellular and extracellular in intestinal tract
Rickettsia ctenocephali Sikora	*Ctenocephalides felis* (Bouché)	Coelomic cavity
Rickettsia trichodectae Hindle	*Trichodectes pilosus* Giebel	Extracellular in intestinal tract
Rickettsia linognathi Hindle	*Linognathus stenopsis* (Burm.)	Extracellular in intestinal tract
Rickettsia culicis Brumpt	*Culex quinquefasciatus* Say (=*C. fatigans*)	In stomach epithelial cells; somewhat destructive to these cells
Rickettsia dermacentrophila Steinhaus	*Dermacentor andersoni* Stiles	Epithelial cells of intestinal diverticula and in other tissues
Rickettsia sericea Gir. & Mart.	*Trombidium (Serico-thrombium) holo-sericeum* (Linn.)	Intestinal tract
Wolbachia pipientis Hertig	*Culex pipiens* Linn.	Gonads of both sexes and occasionally cells of Malpighian tubes
Numerous unnamed species	In both ticks and insects	Usually intestinal tract, although other tissues may be involved

* The term "nonpathogenic" is here used in the sense that the organisms listed are not known to be pathogenic for any vertebrate animal. The same applies to invertebrate animals except that in this case invasion of tissues may occur.

Rickettsia melophagi Nöl. was discovered by Nöller in the sheep ked, *Melophagus ovinus* (Linn.), while studying the flagellates found frequently in this insect. The rickettsiae apparently are present in every specimen and occupy a characteristic position on the epithelial lining of the midgut and probably within certain of the cells (Anigstein, 1927). The rickettsiae are arranged in closely packed rows perpendicular to the epithelial surface. The size of the microorganism averages 0.4 to 0.6 micron in diameter for the coccoid forms and up to 1 micron in length for the more rod-shaped forms. It is gram-negative and has been cultivated on a nutrient-glucose-blood-agar. It also grows well in chick embryos, in which it may be maintained by the serial transfer of infected embryonic fluid.

Wolbachia pipientis Hertig is an interesting rickettsialike organism occurring in the gonads and occasionally in the cells of the Malpighian tubes of *Culex pipiens* Linn. The microorganism may occur in all stages of the mosquito's development. It is a very pleomorphic organism, having various shapes and sizes, both cocci and rods being simultaneously present. Hertig (1936) observed the cells of the gonad wall to contain also an interesting inclusion body which stained brilliantly with neutral red. The relation, if any, of these NR bodies to the rickettsiae is not clear.

One nonpathogenic rickettsia in ticks has been named and studied—*Rickettsia dermacentrophila* Steinhaus, which has been found occurring in the intestinal diverticula and other tissues of the tick *Dermacentor andersoni* Stiles. The organism has not been grown on artificial media but develops well in fertile chicken eggs (Steinhaus, 1942). In the tick transmission apparently occurs via the egg.

These and other nonpathogenic rickettsiae are listed in Table 2.

INTRACELLULAR YEASTS AND YEASTLIKE SYMBIOTES

Earlier in this chapter mention was made of the fact that the true nature of many of the intracellular symbiotes of insects has been difficult to determine with reliable accuracy. Certain forms are relatively large and yeastlike in appearance except that they appear to multiply by fission only and contain no discernible nuclei. Others are slender elongated forms that have nuclear structures and increase by budding. Some have few characteristics of either bacteria or yeasts. Nevertheless certain of the intracellular microorganisms in insects have come to be considered as yeasts or at least as organisms more closely related to the yeasts than to the bacteria. It is these that we wish to consider briefly here.

Nomenclature. Many of the intracellular yeasts have been given names and placed in a tentative classification not clearly integrated with the recognized systematics of yeasts in general. One of the systems for the classification of the yeast symbiotes is that proposed by Brain (1923)

and based on the morphological characteristics of type species as seen in smears and in sections. Since very few of these symbiotes are cultivable on the usual artificial media, the use of cultural characteristics in classifying them is precluded.

By using the suffix *-cola* on the generic name to indicate that the symbiote lives free in the hemolymph or connective fat tissue, Brain sought to distinguish them from those yeasts living in a special mycetocyte or mycetome, in which case the suffix *-myces* was used. Thus we have the following genera:

Lecaniocola	*Cicadomyces*
Kermincola	*Cissococcomyces*
Physokermincola	*Coccidomyces*
Cicadocola	*Icerymyces*
	Aleurodomyces
	Chermomyces

In addition, some forms have been placed in the genera *Coccidiascus, Torulopsis,* and *Saccharomyces.*

In the last-named genus there has tentatively been placed a species (*Saccharomyces anobii* Buchner) associated with the drugstore weevil, *Stegobium paniceum* (Linn.). For the next few paragraphs it will serve as one of the better known examples of the intracellular yeasts found in insects.

The Symbiote of the Drugstore Weevil. *Stegobium paniceum* (Linn.) not only infests many kinds of drugs but also is a pest of cereals and other groceries. Its diet may have caused some of the early entomologists to look for a connection between its food habits and the four peculiar protrusions located at the beginning of the larva's midgut. Microscopic examination of the epithelial cells of these somewhat botryoidal structures revealed the presence of interesting yeastlike symbiotes. Not all the epithelial cells lining these protrusions harbor the microorganisms, only certain large mycetocytes with large jagged-edged nuclei and without a striated border. The adult weevils have similar structures, but they are not well developed.

Within the mycetocytes each symbiote is surrounded by a vacuolated area. The microorganism itself, which Buchner named *Saccharomyces anobii,* is a tear- or pear-shaped cell about 4.5 microns long and 3.5 microns wide. One end is usually pointed, and the other end is broadly rounded; internally it has a refractive nucleus and a large vacuole. It multiplies by budding, with the buds appearing either terminally or slightly to one side of the pointed end. The formation of spores has not been observed. Although some claims have been made concerning its cultivation, most authorities believe that the symbiote has not yet been grown on artificial

media. The symbiotes appear to have some role in the insect's nutrition and perhaps serve as a source of certain vitamins or growth factors. Koch (1933) discovered that symbiote-free larvae do not develop properly unless yeast is added to the diet. Blewett and Fraenkel (1944) believe

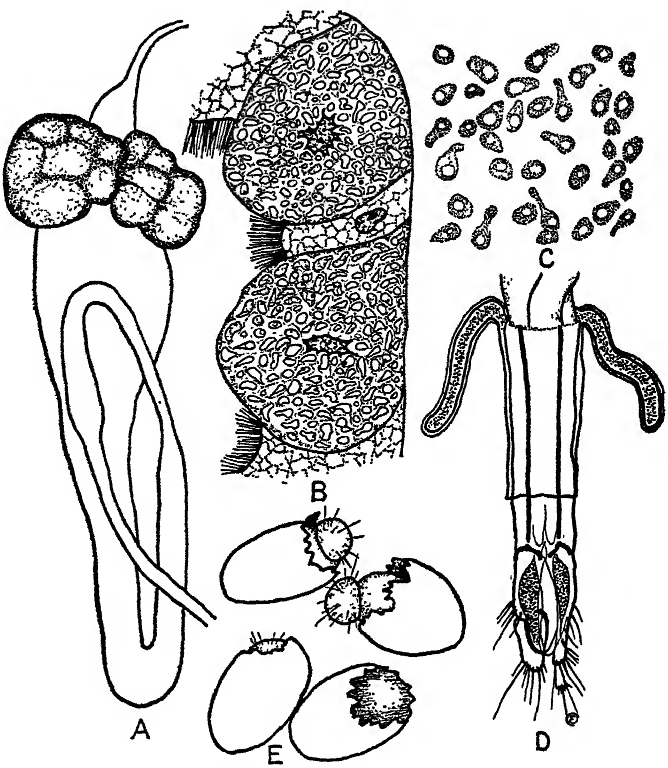


Fig. 51. *Stegobium paniceum* (Linn.) and its yeastlike symbiotes. *A*. Part of the alimentary tract of a larva showing the symbiote-containing diverticular structure attached to the forepart of the midgut. *B*. Section from the wall of the intestinal diverticulum, showing the symbiote-containing mycetocytes between normal epithelial cells. *C*. The yeastlike symbiotes themselves. *D*. Ovipositor of female beetle with symbiote-filled pouches. *E*. Emerging larvae eating parts of the eggshells to which the symbiotes are attached. (*Not to scale. Redrawn from Buchner, 1930.*)

that the intracellular organisms provide the insects with vitamins of the B group, and they present experimental data to support their belief.

The symbiotes are transferred to the next generation in an exceedingly interesting manner. The female adult weevil has two long chitinous pockets, or pouches, located under the vagina. These pouches unite toward the outside opening. In the process of being oviposited, the egg passes down the vagina, past the opening of the vaginal pouches, and

as it does this, the symbiotes are pressed from the pouches out onto the surface of the egg. The symbiotes remain "glued" to the chorion until the larva hatches from the egg. The larva leaves the eggshell head first, and in the process it eats off the edges of the egg opening until about half the egg is consumed. The microorganisms thus gain entrance into the larva. Once within the insect, the yeasts invade the epithelium of the midgut at the site of the future caecumlike structures. The symbiotes

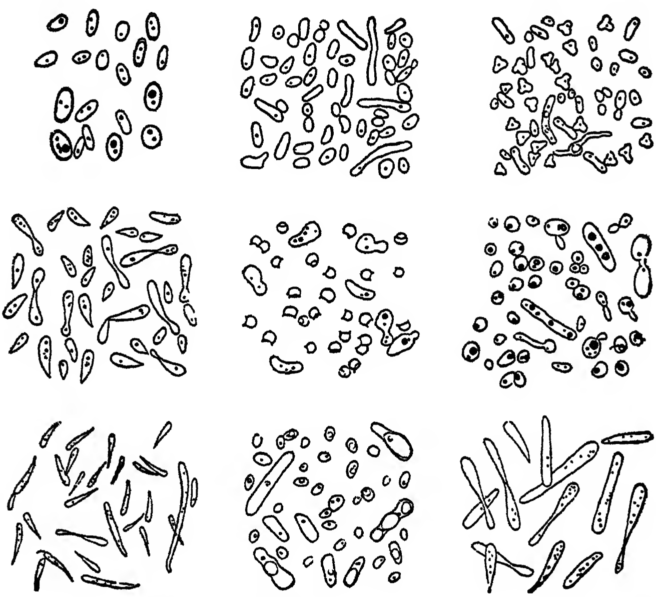


Fig. 52. Yeastlike symbiotes characteristic of various species of Cerambycidae. (*From Buchner, 1930.*)

apparently are introduced into the vaginal pouches of the female during the first defecation of the young adult when they have left the mycetocytes and pass along with the feces.

The symbiotic arrangements in other Anobiidae are in a general way similar to that of the drugstore weevil. The symbiotes are of varied sizes and shapes, but the method of transmission to the next generation is essentially the same.

Yeastlike Symbiotes of Other Beetles. The presence of yeastlike symbiotes among the Coleoptera is probably more general than we now know it to be. In addition to the Anobiidae, with which we have just dealt, the long-horned beetles, or Cerambycidae, have been studied fairly well from this standpoint, particularly the tribes Asemini, Spondylini, Saphanini, Necydalini, Trichomesiini, and Tillomorphini. As a general

rule, those cerambycid larvae which live in the fresh wood of deciduous trees appear to be devoid of the yeastlike microorganisms, while those which live in either living or dead coniferous trees harbor symbiotes (Schomann, 1937).

In cerambycid larvae, the mycetomes frequently consist of small tissue masses or evaginations of the gut wall which circle the midgut in

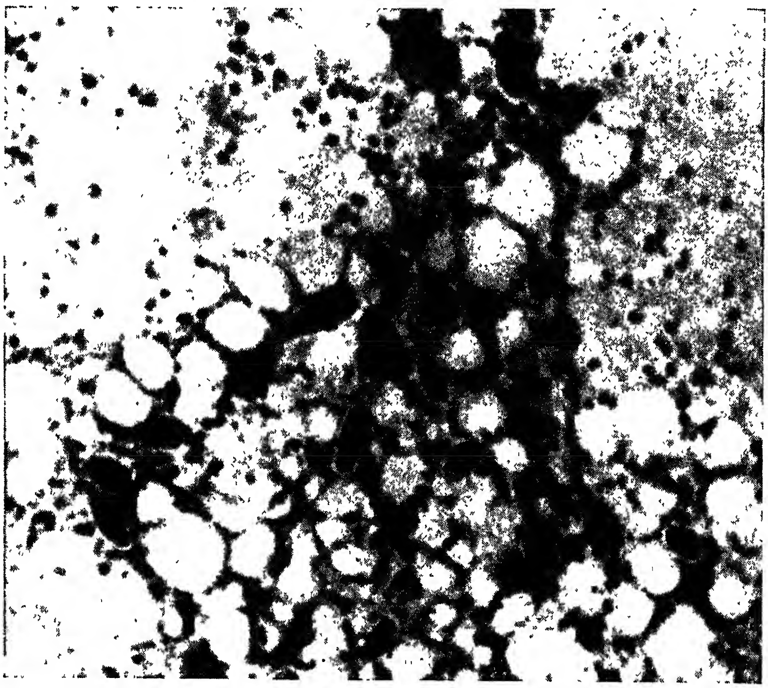


Fig. 53. Yeastlike symbiotes in a stained smear of the body contents of the frosted scale, *Lecanium pruinosum* Coq. (Coccidae). (*Photograph by K. M. Hughes and J. M. Smith.*)

one or two girdles. The cells of the evaginations are filled with symbiotes. When the insects pupate the mycetomes become smaller and the adults have no mycetomes, but the symbiotes are contained in intersegmental pouches of the ovipositor. The symbiotes are smeared on the eggs while the latter are being oviposited. Transmission to the next generation is accomplished in much the same way as has already been described for the drugstore weevil.

Yeastlike Symbiotes of Scale Insects. One has but to make a simple stained smear of the body contents of almost any soft-scale insect to reveal the presence of characteristic yeasts or yeastlike organisms in the hemolymph and connective fat tissue. As a rule the symbiotes are elongated

lanceolate or spindle-shaped bodies varying considerably in size and shape. They are frequently pointed at one end and sometimes at both. In size, they usually range from 3 to 5 microns wide by 6 to 15 microns long. The protoplasm is generally coarse, granular, and vacuolated. Multiplication is by terminal budding, usually at the pointed end. Sometimes the budding forms adhere, forming chains of several buds each, the buds being joined by long necks. Transmission to the next generation occurs via the egg,

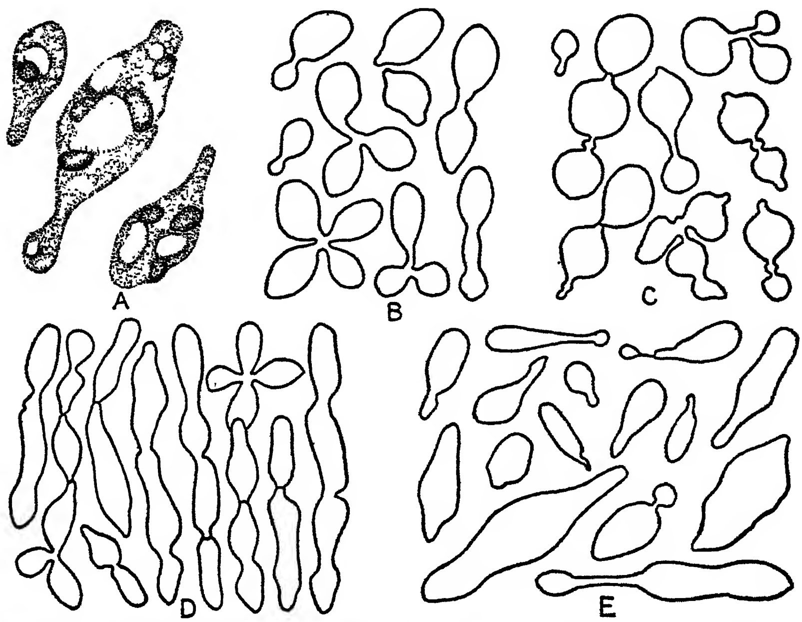


Fig. 54. Yeastlike symbiotes of scale insects. Mostly diagrammatic. *A*. Symbiote from the black scale, *Saissetia oleae* (Bern.), showing protoplasmic contents. (*After Granovsky, 1929.*) *B*. Diagrammatic representation of the symbiote from *Ceroplastodes cajani* Mask. *C*. Symbiote from *Lecanium piperis* Green. *D*. Symbiote from *Lakshadia ficii* Mah. (*After Mahdihassan, 1929, 1935.*) *E*. Symbiote from the nigra scale *Saissetia nigra* (Neit.). (Original.)

the symbiotes, in some cases at least, entering the ovum soon after its differentiation from the nurse cells and the follicular epithelial cells. In a few instances claims of culturing the organisms on artificial media have been made, but such claims need confirmation. The exact role of the symbiotes in the life processes of the insects has not been determined. The use of the symbiotes as an aid in the classification of their hosts has been suggested (Mahdihassan, 1935).

It is a curious and interesting fact that most of the Diaspidae, or armored scales, do not contain these symbiotic microorganisms prominently

in the hemolymph. Instead symbiotes are found intracellularly in definite mycetocytes located throughout the fat tissue. The symbiotes are more rounded or oval than those just described, and the mycetocytes also contain colored or colorless refractive granules and fat droplets. Transmission of the symbiotes is transovarial.

Homoptera other than scale insects harbor yeastlike symbiotes. Among these are certain fulgorids, leafhoppers, treehoppers, psyllids, cicadas, and others. In fact, this group of insects is affording some of the most fascinating observations now being made concerning intracellular symbiosis. Some authors consider the symbiotes of aphids and of mealybugs to be yeasts or yeastlike organisms, but most of the evidence points toward their being bacteria or bacteriumlike organisms, and we have treated them as such earlier in the chapter.

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CHAPTER 6

INFECTION AND EPIZOOTIOLOGY

In the preceding pages it was noted that microorganisms may be associated with insects in harmless commensal relationships or in ways definitely mutualistic. Throughout the remainder of this book we shall be concerned principally with those microorganisms which have harmful influences on insects and which cause infection and disease in their hosts. Nearly all these parasitic organisms have close relatives among the free-living or nonparasitic forms we have been discussing up to this point. Just how or when the various parasitic microorganisms evolved we do not know with any degree of certainty. It is probable, however, that insects suffered from infections and disease long before man began to record the nature of his aches and pains. Nevertheless an accurate idea as to the true cause of infection and disease in insects had to await the understanding of these phenomena in man and other animals.

Theories of Infection and Disease. Toward the close of Chap. 1 it was explained that disease is essentially a process that represents the response of the animal body or plant to injury or insult. It was a long time, however, before this was clearly understood. In the absence of experimentation and controlled observation it was only natural that the early theories of disease were colored by superstition and fear.

One of the earliest theories as to the cause of disease in man was the demonic theory. As concerns the diseases of insects, however, the author knows of no account that dogmatically attributes disease to the activities of evil spirits. Accordingly, we have no authentic record of insect diseases being remedied by exorcising the demons or by frightening the devils away by making terrifying noises or nauseating stenches, although it is just possible that such measures were once used in efforts to save ailing colonies of bees or silkworms.

Among the early theories of disease that have been applied to infectious conditions in insects are the humoral theory and the pythogenic theory. The humoral concept was an outgrowth of the belief of Hippocrates (460–395 B.C.) that diseases in human beings were caused by an imbalance or disharmony of four essential humors: phlegm, blood, yellow bile, and black bile. More frequently, however, the pythogenic theory was employed to explain the ailments of insects. This theory held that disease originated

from decomposition or filth. The existence of disease-producing organisms was appreciated; but, in addition to affording an excellent breeding place, the dirt and filth were considered capable of engendering the infectious agents. Miasmic influences were also frequently blamed for the outbreak of disease, especially in the case of the various maladies of the silkworm. Bad ventilation, certain types of winds, excessively high or low humidities and temperatures, and numerous other agencies were all at one time or another considered to be the specific cause or etiology of insect diseases. We now recognize some of these factors as secondary or contributory causes, but at one time they were considered as primary causes.

/The entire picture changed with the advent of the germ theory of disease—a concept based on the accumulated observations and experiences of many men, including such giants as Fracastorius, Leeuwenhoek, Plenciz, Davaine, Pasteur, and Koch. This theory affirms the definite and specific relationship of microorganisms to infectious disease. And it is well to point out that perhaps the strongest impetus for such an idea arose from the previous discovery that microorganisms were responsible for fermentation, putrefaction, and decay.

INFECTION

Infection (L. *inficere* = to put into, to soil, or to stain) is a biological relationship, resulting in disease, in which an invading microorganism settles, grows, and multiplies in the tissues or body fluids of the host organism. It is comprised of two main factors: invasiveness and the initiation of a disease (or an impairment of bodily functions). An *infectious disease* is simply a disease due to the presence of a living organism. When an infectious disease is naturally transmitted by direct contact, *i.e.*, when it is "catching," we speak of it as a *contagious* or *communicable* disease. Infection is to be differentiated from *contamination*, which is merely the harboring of or contact with microorganisms not normally in this association. It is a term ordinarily used in reference to inanimate objects or cultures. Thus a dissecting instrument, a tube of media, or one's hands may be contaminated although not infected. An *infestation* is a situation in which considerable numbers of a parasite enter or attach themselves to their host, although this may never overbalance the host-parasite relationship. For example, a person may be infested (not infected) with lice, or an insect may be infested with mites. An *intoxication* is manifest by the presence of symptoms caused by the activity of microorganisms, although the microorganisms themselves are not necessarily present in the afflicted individual. An example of this is botulism in man, in which infection by the bacillus is of no danger but the consumption of the toxins produced by the bacillus in improperly canned foods is. Intoxication can

occasionally take place in insects, as will be brought out later in our discussion of *Bacterium entomotoxicon* (Duggar).

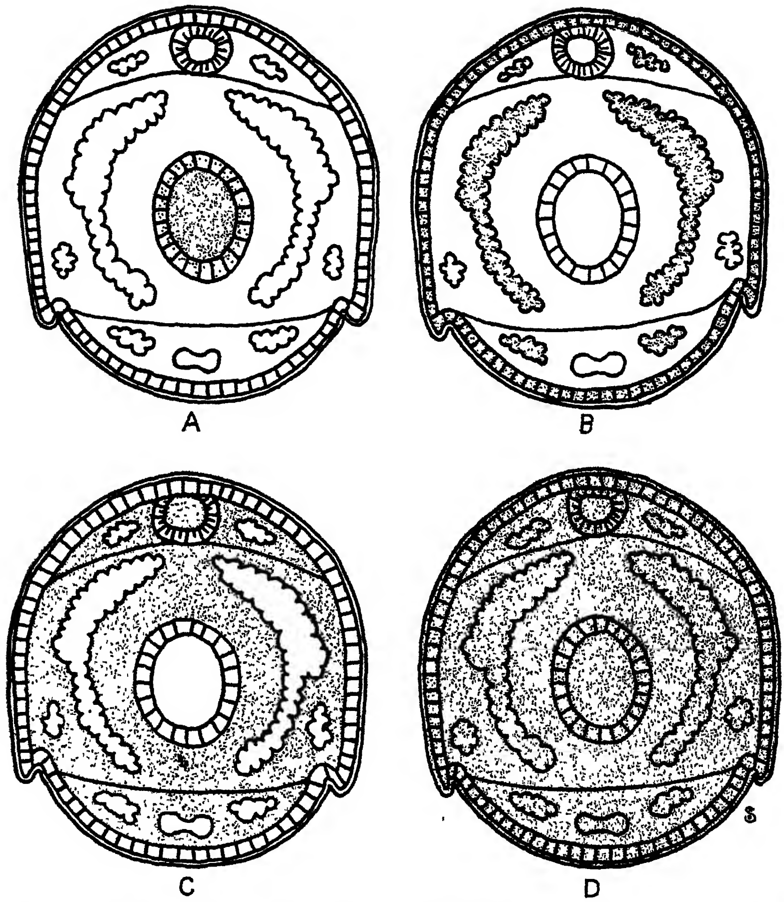

Fig. 55. A diagrammatic representation of the general types of infection occurring in insects as visualized from a cross-section aspect. *A*. The intestinal, or dysentery, type of infection in which the invading organism is limited to the alimentary tract and its appendages. *B*. Tissue infection, here indicated by the stippling of the adipose tissue and the hypodermis. *C*. Septicemic type of infection in which the invading organism multiplies in, and is distributed throughout, the body cavity by the hemolymph. *D*. A general systemic infection in which the invading organism penetrates to all parts of the insect's body.

Infectious agents are included among each of the principal groups of microorganisms: bacteria, yeasts, molds and higher fungi, viruses, rickettsiae, spirochetes, and protozoa. It should be remembered, however, that the majority of these forms of life are not infectious. Certain micro-

organisms may be parasitic on or in the insect body and yet not be pathogenic, *i.e.*, cause disease. Others, as we have explained earlier, may be merely casual associates in a purely adventitious relationship, and still others may be actually beneficial to the insect that harbors them.

Kinds of Infection. A varied terminology is employed in company with the use of the term "infection." The several kinds of infection may be classified according to

1. The extent of the infectious process in the host (*e.g.*, local, focal, and general or systemic)
2. The site of the infection (*e.g.*, intestinal, fat body, blood)
3. The course of the disease (*e.g.*, acute, subacute, chronic, latent)
4. The source of the infecting agent (*e.g.*, exogenous, endogenous, and idiopathic, or hidden)
5. The type of etiological agent (*e.g.*, bacterial, protozoan, fungous, virus)
6. The distribution or extent of the infection in the insect population (*e.g.*, sporadic, enzootic, epizootic)
7. The mode of transmission (*e.g.*, food-borne, water-borne, direct contact, fomites)
8. The basis of sequence (*e.g.*, primary, secondary, mixed or multiple, terminal)

Virulence. It is difficult to define the term "virulence" in a precise way and have a definition satisfactory to everyone. Perhaps it is most convenient to define virulence as the disease-producing intensity or power of a microorganism, *i.e.*, the ability of a microorganism to invade and injure the tissues or body of its host. The ability of some microorganisms to form toxins is included in the definition by some authors. In any case it is a relative term with respect to the host, since it is obvious that a weakened host is more susceptible to a microorganism of given disease-producing power than is a host offering a high resistance. Nevertheless the term "virulence" is usually used to designate an attribute of the microorganism, as distinguished from the host's resistance or susceptibility. The process of increasing the resistance of the host is called "immunization"; that of decreasing the disease-producing power of a microorganism is called "attenuation."

The virulence of a microorganism pathogenic for insects may, in general, be increased in several ways, including (1) passing it through insects (or possibly other animals); (2) causing it to dissociate into its more virulent and less virulent strains; (3) introducing, together with the microorganism, substances (mucin, starch, etc.) that may aid in increasing its invasive powers; (4) associating it in a mutualistic relationship with other microorganisms that may render it more capable of invading tissues than it would be otherwise. On the other hand, the virulence of an organism may frequently be decreased by (1) passing it through insects or animals unfavorable for its growth and development; (2) causing it to

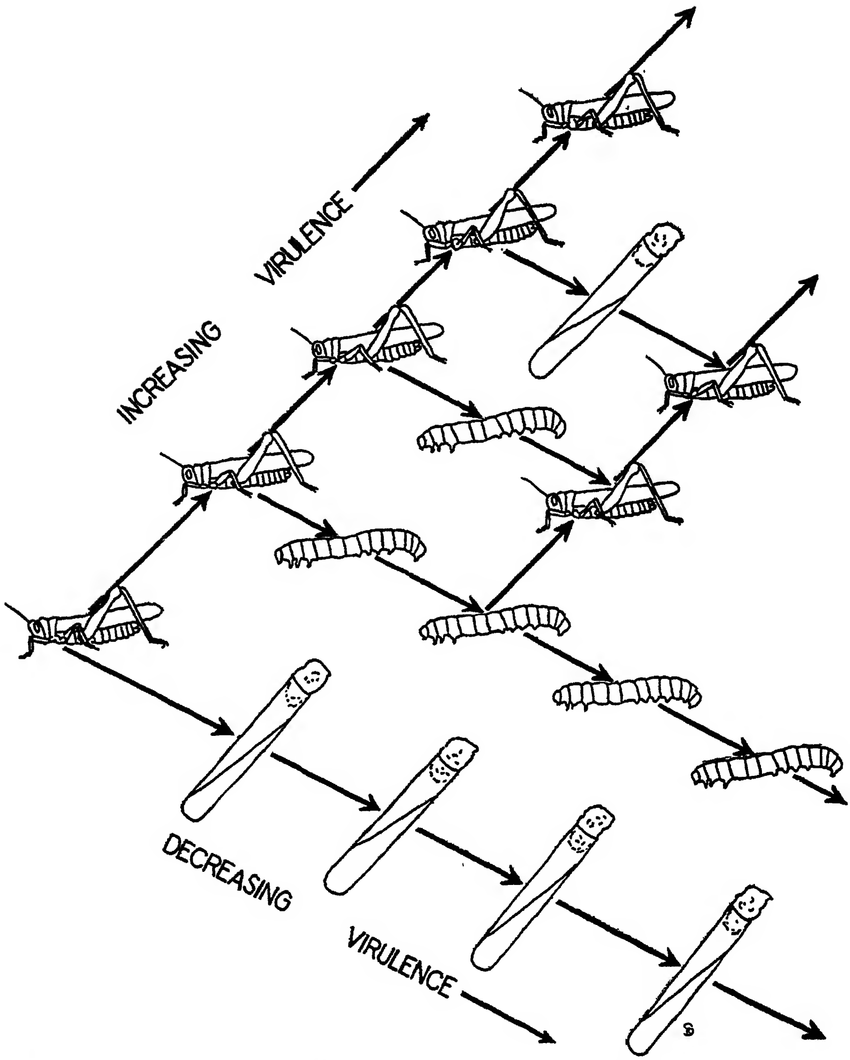

Fig. 56. A diagrammatic representation of manners in which the virulence of a microorganism may be increased or decreased for some insects. As indicated, a microorganism, *e.g.*, a bacterium, that is moderately pathogenic for grasshoppers, may frequently have its virulence for grasshoppers increased by passing it several times through this host animal. Up to a certain point, its virulence enhances with each passage. Conversely, if the bacterium is passed through a nonsusceptible host (in this case represented by a caterpillar), or if it is passed through successive transfers on artificial media (represented by test tubes), its virulence for grasshoppers may decrease. If, after this treatment, the bacterium is again passed through grasshoppers, its virulence for this insect is likely to increase again.

dissociate into strains of low and high virulence; (3) cultivating it at abnormally high temperatures; (4) cultivating it under abnormal nutrient conditions.

The matter of increasing the virulence of entomophytic bacteria by repeated passages through susceptible insects is not, however, a phenomenon characterized by the constancy that is found in the case of the virulence of vertebrate pathogens. Paillot (1933) has presented an analysis of data that indicates that the virulence of some entomophytic bacteria, for any given species of insect, varies greatly from one individual to another. The virulence does not usually follow a regular progression according to the number of passages through individuals of the same species. In fact, the virulence may decrease as suddenly or as gradually as it increases. The factors that determine the direction and amplitude of these irregular variations are unknown. Nor is it known why this seems to be in contradiction to the state of affairs as it occurs in vertebrates, except that profound anatomical and physiological differences in the two groups of animals must be recognized to have some possible connection with it.

As far as its use in this volume is concerned, the term "pathogenicity" may be considered synonymous with virulence. A pathogen (Gr. *pathos* = suffering + *gen* = producing) is simply a microorganism that will cause an infection or disease in a particular animal or plant. Pathogenic organisms may, for convenience, be grouped into two categories: the "opportunists" and the "true" pathogens. Opportunists are those microorganisms which live in constant association with the host, such as those in the alimentary tract. Under certain conditions the barrier or resistance that usually protects the host may be broken down, enabling the microorganisms to invade the more susceptible parts of the insect's body. Sometimes they invade the body tissues only when a special opportunity is afforded them by some preliminary infection or injury. In such cases they are often referred to as "secondary invaders." This does not necessarily mean, however, that they are of secondary importance. Unlike the opportunists, the true pathogens are capable of invading under normal conditions of host resistance and rarely live in close association with the insect without producing disease.

Other Factors Concerned in Infection. As in other animals, the infectious process in insects embodies numerous factors that influence the nature or the progress of the infection in some way or other. A detailed treatment of these factors will be found in any good textbook of medical bacteriology or pathology. Here we can mention only a few of them very briefly.

One of these factors concerns the *portal of entry* by which the invading

microbe enters the body of the insect. Disease-producing microorganisms frequently have some special part (or parts) of the body that affords them ready entrance into the insect's body. This vulnerable point may be the integument, broken or intact, the intestinal tract, the spiracles, or other body openings. The portal of entry may, in general, vary also according to the group of microorganisms concerned. Thus most bacteria and viruses enter the insect body by way of the mouth and intestinal tract, whereas most fungi enter the body cavity by penetrating the integument or body

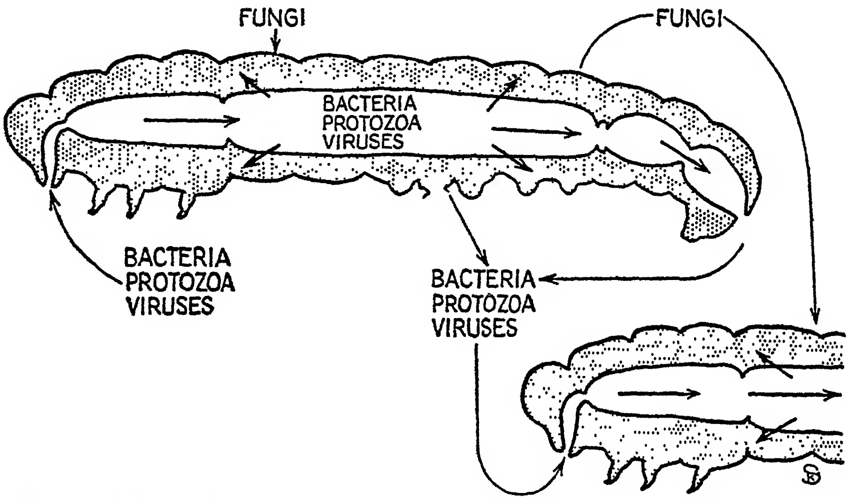

Fig. 57. Diagrammatic representation of the several routes by which infectious microorganisms can gain entrance into an insect host and be disseminated again.

wall of the insect. Sometimes particular parts of the integument are more vulnerable than are other parts, but frequently no such specificity exists.

The portal of entry is often associated with the particular manner in which the pathogenic microbes are discharged from the body. Agents for which the mouth is the portal of entry are frequently liberated from the anus along with the intestinal discharges of the insect. This characteristic permits the food of healthy insects to become contaminated, thus perpetuating the infection. The spores of a fungus developing in a diseased insect are disseminated in such a way that they come into direct contact with the integument of a healthy specimen, which is then invaded. A sound knowledge of the portals of entry and modes of discharge of the microbes in infectious diseases always enhances our knowledge and understanding of the disease as a whole.

Another important factor in infectious processes concerns the number of microorganisms involved. To begin with, it usually takes a certain

number of organisms to overpower the immediate or local defenses of the body and initiate the infection. If the organism is able to multiply rapidly, yielding large numbers, the infection is likely to be more severe and have a shorter incubation period than would be the case if smaller numbers developed.

The time elapsing between the entrance or introduction of the microorganisms into the insect body and the development of symptoms is known as the *incubation period* of an infection or disease. It is during this time that the microorganisms are multiplying and producing their poisons. The *beginning of disease* includes the period from the first appearance of symptoms until they are fully developed. Clinically, this is the period from the start of the infection until it reaches its greatest severity. The *height of disease* includes the period from the time the symptoms attain their full height until they begin to fall. During this period the microorganisms are most active, their toxins are present in the greatest amount, the lesions caused by them are greatest in extent, and the symptoms are the most severe. Ordinarily the end result of a frank infection is either *recovery* or *death.* If the infecting microorganism overpowers the defensive mechanisms of the insect, death is the likely outcome. If the insect is able to ward off or control the infection, recovery is the rule.

Three other terms frequently used in speaking of infections deserve definition. When bacteria are present in the hemolymph or blood of an insect, as of other animals, but are producing no harmful toxins or other deleterious effects, the condition is called a *bacteremia.* When the microorganisms actually multiply in the blood and thereby bring about harmful reactions, the condition is known as a *septicemia.* In the case of most insects this implies an infection of the entire body simultaneously. A *toxemia* is a condition produced by the dissemination of bacterial toxins or other poisonous substances in the blood.

At this point it might be well to deal with certain terminology that applies specifically to infections and parasitizations in insects. It is well for the student in entomology, as well as in insect pathology itself, to be able to keep the meanings of these terms clearly differentiated; otherwise they are likely to be confusing to him. Since the word *entomic* is an adjectival form relating to insects it can frequently be used in a convenient general sense as, for example, "entomic bacteria." The term *entomogenous* simply means the growing in or on the bodies of insects (*e.g.* "entomogenous fungi"). It usually connotes an intimate or parasitic relationship. This term should not be confused with *entomophagous,* which means insectivorous and ordinarily refers to the eating and ingesting of insects. It is usually applied not to microorganisms but rather to those insects (and other animals) which parasitize or eat other insects. *Entomophytic* is essentially syn-

onymous with "entomogenous." It may be used to refer to almost any relationship between plant microorganisms (bacteria and fungi) and insects. The word should not, however, be used when referring to protozoa. The microorganism itself may be referred to as an *entophyte* or *entomophyte*, which simply means that it is a plant (such as a parasitic bacterium or fungus) living within or on the body of the insect. The term *entomophilic* ("insect-loving") is also used to cover the associations not only between insects and plant microorganisms but also between insects and protozoa and between insects and nematodes.

Koch's Postulates. In the early days of bacteriology numerous bacteria were isolated from various disease conditions in man and other animals, and without much more ado they were named as the cause of this or that disease. It soon became evident, therefore, that more exact methods of isolation and identity were necessary. The first noteworthy attempt to standardize the procedure for furnishing unequivocal proof of a suspected causal relation between a given microorganism and a particular disease was provided by the bacteriologist Robert Koch (1843–1910). The formulated chain of experimental evidence that Koch considered necessary for this proof is often formalized as a series of postulates, which, when applied to human infections, are commonly known as "Koch's postulates." They may be expressed as follows:

1. The microorganism must be present in every case of the disease.
2. The microorganism must be isolated in pure culture.
3. The microorganism in pure culture must, when inoculated into a susceptible animal, give rise to the disease.
4. The same microorganism must be present in, and recoverable from, the experimentally diseased animal. (Sometimes certain serological correlations are also required.)

If the above steps are carried out, the evidence implicating the microorganism as the causative agent is certainly very strong. Situations occur, however, in which one or more of these steps cannot be followed or can be followed only with great difficulty. For example, many viruses do not produce inclusion bodies and hence cannot be observed in the tissues of the infected animal by ordinary microscopic methods; their presence, however, can usually be detected in other ways. Furthermore, some microorganisms have not yet been successfully isolated in pure culture, a fact that would necessitate the circumventing of postulate 2. In the case of many insect diseases postulate 3 must be watched carefully, since different microorganisms frequently produce the same or similar symptoms (*e.g.*, septicemias); in addition, the symptoms that appear in nature may be different from those produced in the laboratory with the same microorganisms.

How Microorganisms Produce Infection. Two mechanisms are involved in the production of an infection or disease as caused by most microorganisms. These are the microbe's production of chemical or toxic substances, and the causation of mechanical destruction. In the latter category are included such effects as the trauma and mechanical pressure brought about in the host's tissues by the sheer growth and development of the parasite, which may cause infected cells not only to hypertrophy but to burst or become disrupted. The infecting microorganism may also bring about some ill effects by the mechanism of utilizing the critical food elements circulating in the hemolymph, or that of utilizing the oxygen supply in certain of the insect's tissues. In addition to the two mechanisms mentioned there may, of course, be a third that consists of a combination of the chemical and the mechanical factors.

In infections in vertebrates we have a fairly accurate idea as to the role of the chemical or toxic substances produced by microorganisms, particularly bacteria, in the causation of disease. With some justification, we can probably assume that their role is much the same in insect diseases. Accurate experimental data on this point, however, are very meager except in a few of the infections in insects studied. Most of the information at hand concerns bacterial infections rather than those caused by protozoa, viruses, or fungi.

The chemical or toxic substances produced by most bacteria may be separated into two general types, depending on the manner of their production. Thus we may have *catabolic* substances, which are the results of decomposition brought about by the activity of the microorganism and which may arise from either the substratum upon which they are living or from the decomposition of the bodies of the microorganisms themselves. Thus there may be produced certain acids, alcohols, mercaptans, alkaloids, other protein cleavage products, and the like. Just how often or in what ways these catabolic substances produced by microorganisms affect insect life is not well known. The second group of chemical substances concerned are *anabolic* substances, which are toxic or destructive substances synthesized by the bacteria. A number of these substances have been studied in considerable detail, particularly with respect to vertebrate infections. Perhaps the best known of these are the exotoxins and endotoxins.

Exotoxins (also called "ectotoxins," "true toxins," and "soluble toxins") are toxic or poisonous substances produced by the microbial cell and liberated into the surrounding medium outside the cell. Exotoxins are produced by plants (phytotoxins) and animals (zootoxins) as well as by bacteria and other microorganisms. Bacterial exotoxins may be produced wherever the microorganism grows well, in vivo and in vitro. Although these substances have not received adequate attention in insect

pathology, most of us are familiar with classic examples of toxin-producing bacteria in human pathology, such as the bacilli of botulism, diphtheria, tetanus, and dysentery. The principal characteristics of exotoxins, which as poisons are more powerful than chemical poisons, include the fact that (1) they require an incubation period before their action is apparent, (2) they give rise to the production of antibodies (antitoxins) which are able to neutralize the toxins, (3) they are extremely labile, (4) they are soluble in water, and (5) they are apparently protein in nature.

Endotoxins or endotoxic substances are not secreted into the surrounding medium but are confined within the microbial cell. Many authors use the term "endotoxin" merely as a collective name for the cause of toxic reactions obtained when dead bacteria or mixtures of bacterial substances are injected into an animal. We really have no clear understanding as to the true nature of endotoxins, or even whether or not they are actually "toxins." They appear to be intracellular constituents of the microbial cell that are not set free during life but may be set free upon the death and dissolution of the cell. In a sense it may be said that endotoxins are present in all bacteria capable of producing disease. Other characteristics of endotoxins include the fact that (1) they are less diffusible than exotoxins, (2) they are very stable in presence of heat and certain chemicals, (3) they have a low degree of toxicity as compared with exotoxins, and (4) they do not stimulate the production of antitoxins.

Other anabolic substances that are produced by bacteria and aid these microorganisms in producing disease include such substances as lysins, necrotoxins, capsules, leucocidins, and spreading factors (*i.e.*, substances that increase the permeability of tissues). In general, the injurious substances produced by bacteria are usually of the type the actions of which appear to be directed against the defensive mechanisms of the host or which destroy tissue or impair its capacity to function.

EPIZOOTIOLOGY OF INSECT DISEASES

Epizootiology is the science that seeks to explain infectious diseases of animals on the basis of mass phenomena; *i.e.*, it is concerned with diseases as they occur in groups of animals rather than in the individual animal. It may also be thought of as being concerned with the natural history of infectious diseases among animals. In a narrow sense the word "epizootiology" is sometimes used to refer only to those phenomena associated with epizootics as distinct from those associated with interepizootic or with enzootic periods. Most authorities, however, use the term in a broad sense, referring to the enzootic as well as to the epizootic phenomena of infectious diseases. The word "epizootiology" has essentially the same

meaning in regard to other animals as the word "epidemiology" has in regard to human beings.

Terminology. As in the epidemiology of human diseases, so with the epizootiology of animal diseases: a particular terminology is employed to facilitate a common understanding of the factors involved. It is well that we define some of these terms here. Most of them are borrowed from the literature on human epidemiology.

We have already used the terms "epizootic" and "enzootic," and their meanings are probably clear to most of the readers of this book. Suffice it to say that an *epizootic* disease is a disease or a phase of a disease of high morbidity and one that is only irregularly present in clinically recognizable form; an *enzootic* disease is one that has a low incidence but is constantly present in a population. "Epizootic" is analogous to "epidemic." The student may be helped in accepting this apparent superfluity of words if he remembers their Greek derivations to the effect that etymologically *epidemic* means "on the people," while *epizootic* refers to animals and, of course, could be used broadly to include the human animal. The word epidemic, however, is so well established and understood as to warrant its distinctive use in reference to the diseases of human beings.

Morbidity refers to sickness or disease; *mortality* refers to death. Morbidity statistics include cases that recover as well as those that die; mortality statistics are concerned with fatal cases only. These statistics are frequently expressed as *rates*, or the number of cases or deaths occurring in a population of a certain size over a certain period of time. In insect pathology, when it is desired to make comparisons over relatively long periods of time, the mortality rates are usually much more significant and accurate than are the morbidity rates. This is especially true since the severity of a disease among insects is usually ascertained by determining the number of dead insects in a population rather than the number of sick individuals. A rate known as the *proportionate* or *percentage mortality* is sometimes used to express the proportion that the deaths from any given disease bear to the total deaths from all causes.

Rates expressed in terms of all stages of all the insects in a population are called *crude rates*. However, since some diseases are most prevalent in larvae, some in pupae, and some in adults, and since there are other more intrinsic differences in the individuals or groups making up any population, it is necessary to make corrections for these differences if we are to have a true picture of the disease. Sometimes it is necessary to make corrections for varieties or races of insects before comparing morbidity and mortality rates. To assist in making any of these corrections, specific or standard rates are used. *Specific rates* are expressed in terms of the proportion of cases or deaths in a particular instar or stage of the insects

concerned. *Standardized* rates are expressed in terms of a standard population of the insect in question in a definite area; *i.e.*, the approximate annual population in a given area may remain more or less constant year after year, and if the density of the population is once determined it may, with caution, be used as a standard for determining morbidity and mortality rates.

The term *case fatality rate* refers to the percentage of deaths, *i.e.*, to the number of deaths in every 100 cases of a particular disease. Such information is frequently of not much practical value in insect diseases since recovery in insects is either very rare or is very difficult to determine. In the majority of cases, if an infected insect shows recognizable signs of disease, it will succumb. It is sometimes useful, however, to determine the case fatality rate at a particular time in an epidemic even though it is known that practically all the sick individuals will die rather than recover.

Incidence refers to the degree of occurrence of a disease in a particular population or, in other words, the ratio of diseased individuals to healthy individuals in a given population. Thus we speak of a high or low incidence of a disease, meaning that a large or small portion of the population is infected. The *prevalence* of a disease is a function of its incidence times its duration.

It will be noted that these terms are all applicable to groups of insects rather than to the individual insect. For the "clinical" description of a disease, the unit is an individual; for the epizootiological description, the unit is an aggregation of individuals or *population*. The term "population" may refer to a group of insects that collectively inhabit an area or region, or to the entire group of insects from which samples are taken for measurement or examination. The economic losses caused by insects are due primarily to their activity as populations since individually these small creatures are ordinarily quite harmless. Similarly the effectiveness of various control procedures is ascertained in terms of populations rather than in terms of individual insects.

The Epizootiological Method. In studying the epizootiological aspects of a disease, the insect pathologist must gather his data from various sources, add this information to his own observations, arrange in a logical manner the data obtained, analyze and interpret the data statistically and otherwise, and finally must make his conclusions as to the significance of his data and observations.

Unfortunately no reliable reporting of insect diseases is maintained on a widespread or thorough basis, as is the case with human diseases. Furthermore, most of the outbreaks that are reported are not adequately studied or followed to their ultimate conclusions. Occasionally, however,

it is possible to obtain sufficient supporting information from the observations of different workers in several different areas to make conclusions or interpretations on an epizootiological scale. The most effective "data gathering," however, is that which is accomplished by a single organized group of workers who are simultaneously able to extend their observations over the entire area concerned.

The character of the data obtained may vary somewhat according to the nature of the disease, but in general it will consist of information concerning the geographical extent of the disease; the species of insect or insects involved; the age, stage, and sex of the diseased insects; the date of onset and duration; the prevailing climatic conditions, especially as they pertain to temperature and humidity; the food plant of the host; and any other information that may be deemed pertinent.

In attempting to analyze the data in order to find the common factors, considerable reliance must frequently be placed on the use of statistical methods. Some idea as to the application of the statistics of averages, dispersion, frequency distributions, probability, correlation, regression, and forecasts should be had by the insect pathologist if he is to analyze properly all the data he may collect in his epizootiological studies. The cautious and judicious use of statistics is a very important part of the science of epizootiology; and, although an unscrupulous statistician may appear to make his statistics prove almost anything, it is nevertheless certain that without the use of statistics and sound logic the epizootiologist is seriously handicapped in understanding the phenomena with which he deals.

Primary Factors in Epizootics

Epizootics, like epidemics, are concerned with three primary factors: (1) the infectious agent with its variable virulence and infectivity, (2) the susceptibility or resistance of the individuals that compose the population at risk, and (3) an efficient means of transmission. To the sum-total effect of these three factors affecting the spread of any specific infection at a given time and place, Stallybrass (1931) has given the title "dispersibility."

Each of these primary factors varies greatly and is influenced by certain intrinsic and external secondary factors. Although each of them may operate separately, it is more characteristic of them to operate together and to be closely interdependent. In fact, the first two of the primary factors listed above are so frequently and so closely interrelated that it is convenient to discuss certain aspects of them together.

In addition to the type of variation just mentioned are the variations in disease prevalence that will be mentioned later in this chapter.

Effect of Microbial and Host Variations on Epizootics. In epidemics of infectious disease among human populations it is well known that variations in the virulence of the different strains of the infecting microorganisms may markedly affect the course of the epidemic. In fact, some strains of bacteria and viruses characteristically are known as "epidemic" strains; others are of such a low virulence that they give rise to very mild or transient cases that rarely reach epidemic proportions. On the basis of remarks we made earlier in the chapter we may also consider the increased virulence of microorganisms as representing a decrease in host resistance. In actuality, a fluctuating equilibrium is maintained between parasite and host, and we must be careful to differentiate between a change in this balance and a true rise or fall in virulence on the one hand, and a true increase or decrease in the resistance of the host on the other. In entomogenous bacteria it has been demonstrated that the virulence of the particular strain concerned may frequently be enhanced by repeated passage through susceptible hosts. This effect has been seen both in the laboratory and in the field where the intensity of the epizootic increases along with the virulence of the infecting organism. By and large, however, we know very little about the effect of microbial variation in virulence upon the infection in an insect population. We have, for example, no clear-cut picture of the differences, if any, in strains of the same bacterium isolated at different periods in the same or in different epizootics, or of the relation of these changes to fluctuations in mortality that are observed in long-continued epizootics. Most animal and plant viruses exhibit striking degrees of variation, resulting in the existence of numerous strains of varying virulence. Similar variations have not been generally recognized in the case of the insect viruses, but that such exist is probable.

As to changes in host resistance, on the other hand, we have slightly more information on which we may deliberate. Factors involved here include the effects of such things as adverse conditions of temperature, humidity, and nutrition, and the presence of host immunity. Of course, such circumstances may also affect the microbial parasite, but actual experimental data on this point are at hand in only a few instances.

The effects of various environmental factors on host resistance, however, are difficult to separate from those which affect neither the host itself nor the microorganism itself but which affect the host-parasite complex as such. In other words, the infection itself may be influenced by certain conditions that would influence either the host or the parasite separately very little.

In the next few pages we shall briefly consider some of these factors which may affect either the infectious microorganism, the host, or the host-parasite complex. Our discussion, however, will deal mainly with

these factors as they affect whole insect populations rather than isolated individuals.

Population Susceptibility and Immunity in Relation to Epizootics. In the past most of the observations relating to the susceptibility and resistance of certain species of insects to certain microoganisms have been made on the basis of individual insects or small groups of insects. If we are to understand the true nature of epizootics and their spread among insect populations we must take into consideration what epidemiologists of human disease call "herd infection" and "herd immunity" (Topley, Wilson, and Miles, 1946). Since large numbers of insects are usually known as "populations" we may use the terms "population infections" and "population immunity" in speaking of these factors as they relate to the diseases of insects.

A population, like each of its individual members, has a characteristic composition or structure. This structure may include, besides the individual members with their spatial relationships to one another, alternative hosts with various types of distribution, and all those environmental factors which favor or inhibit the spread of the infection from host to host. In addition, a population may be "immune" to a particular disease in the sense that it will resist the introduction of infection from without, although each of its members is fully susceptible; or the population may be so situated that it is not subject to infection. If any of the individuals were to stray to a population with a structure that permitted the disease in question to exist in endemic form, it would probably become a victim to infection. Thus an insect living in a regularly arid locality is not subject to attack by such microorganisms as entomogenous fungi, which flourish in warm humid areas; the susceptible insect, however, might be readily attacked if it migrated to an area in which the optimum conditions for the growth of the microorganism existed. One of the principal hopes of bringing about a degree of microbial control in an insect population lies in our ability to bring about a change or alteration in the population structure; but to interfere with the course of events intelligently one must understand the details of the factors involved.

Let us now consider the various types of individuals that may go to make up the membership of a population. Topley and Wilson (1936) list six theoretical categories of hosts among any infected human population: (1) the typical case, (2) the atypical case, (3) the latent infection, (4) the healthy carrier, (5) the uninfected immune, and (6) the uninfected susceptible. As concerns most insect populations, for all practical purposes these may logically be reduced to (1) the typically diseased insect, (2) the atypically diseased insect, (3) the uninfected immune, and (4) the uninfected susceptible. The presence and status of individuals having

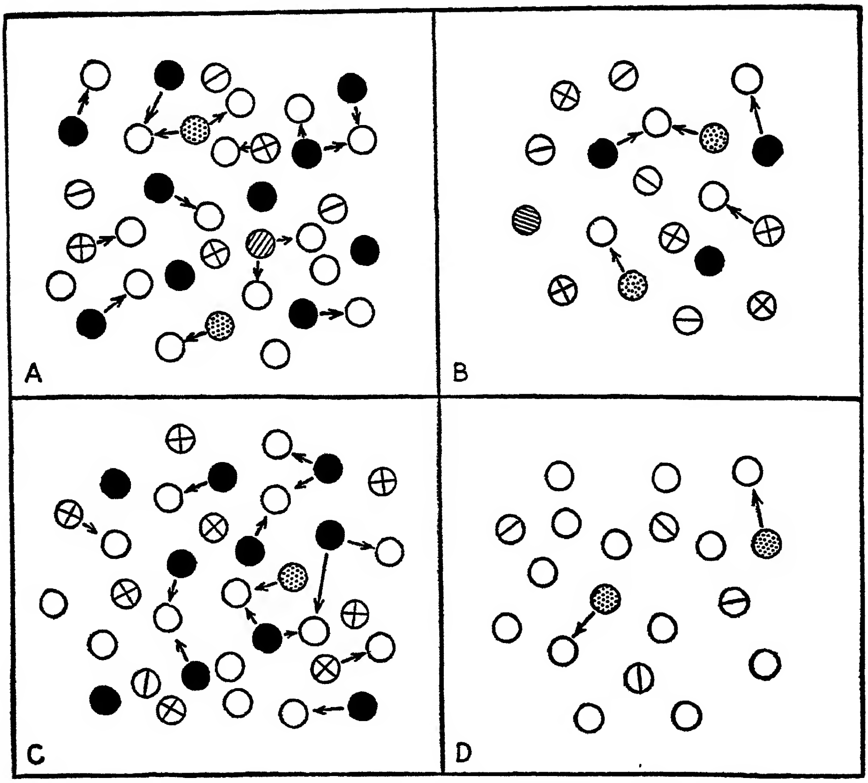

Fig. 58. Diagrammatic representation of the types and distribution of individuals that may exist in infected populations of insects under different epizootic conditions. *A*. The situation during an outbreak (epizootic) of an infectious disease from which the affected population is never completely free. *B*. A later stage of *A* in which there is a small epizootic wave and in which the number of susceptibles are few. *C*. A severe epizootic occurring in a population with little initial immunity or over-all resistance such as might occur when a disease agent is introduced into a new, highly susceptible population for the first time. *D*. The relative quiescence that may occur between two outbreaks of the type indicated in *A*. ● typically diseased insect; ◍ atypically diseased insect; ◌ healthy carrier; ⦸ uninfected immune; ○ uninfected susceptible; ⊗ insect killed by disease; → direction of effective spread.

latent infections and individuals that act as healthy carriers are inadequately known in insect populations. Probably such do exist, and it is not unlikely that some of the infecting agents are held over the periods between epizootics in living insects that thus may act as carriers. Theoretically, therefore, a fifth group may provisionally be added to include the individuals which may have latent infections or which may act as healthy

carriers—two qualities essentially the same. In addition to all the five groups is that part of the infected insect population which has been killed off by the pathogen. Since most disease epizootics in insect populations are characterized by the number of individuals they destroy, this group is more significant in our present considerations than is frequently the case in human epidemics. Not infrequently, through cannibalism or contact, these dead insects serve as foci of infection from which the pathogen may effectively spread to healthy individuals. Following the method used by Topley and Wilson, we may illustrate the manner of distribution of the five types of individuals which may be found in infected populations under different epizootic conditions by the accompanying diagram (Fig. 58).

In many human infections the immune individuals greatly outnumber the susceptible ones. There is no proof that such is the case with regard to insect infections; in fact, indications are that the opposite is true; *i.e.*, when a species of insect is known to be naturally susceptible to a particular disease agent, the susceptible individuals usually greatly outnumber those which are immune. Whether this situation is changed during the course of most epizootics we do not know. There appears to be very little evidence as to whether or not the spread of infection results in the immunization of most of the surviving insect population. In human epidemics, the survivors are, on the average, more resistant than are newcomers to the group.

Infectivity, or Capacity to Spread. Infectivity, or the capacity of a pathogenic microorganism to spread from one insect host to another, is one of the most important factors concerned in any epizootic within an insect population. Naturally this capacity may vary according to the particular conditions prevailing and according to the exposure of the susceptible insect to risk of infection.

Direct contact between infected and healthy insects is an important means of spread in the case of fungous diseases in which the dead insect supports germinating hyphae and fruiting bodies, and in any disease which may be acquired through the cannibalistic habits of insects. The closeness and extent to which insects come together in the course of their activities may be termed *aggregation*; the opposite of this, and used in a static sense, is *dispersion*. The word *dispersal* has been used in an active or dynamic sense to mean the extent to which insects leave their accustomed habitat or area and come in contact with fresh populations.

As stated by Stallybrass (1931), the maximum opportunity for the spread of infection will occur when a center of close aggregation is associated with marked dispersal. Such a center becomes a nodal point from which lines of communication radiate. The influence of aggregation

is not so great in instances in which the disease agent is carried by flying or actively moving insects as it is in cases in which the insects have opposite habits of movement. When disease agents depend upon direct contact for their dissemination, it is usually true that, other things being equal, the greater the number of contacts the greater the chances of infection.

In general, there appears to be a definite relationship between the dosage of microorganisms and the proportion of deaths that follow. The susceptibility of some insects to certain infecting agents is so high that a high fatality rate follows the inoculation of extremely few microorganisms—possibly only one being necessary in some cases to produce infection. In most instances a "critical dose" is probably necessary to overcome the resistances offered by a particular insect to a particular microorganism.

It is reasonable to suppose that, with most insects, doses smaller than the minimal infective dose (M.I.D.) are destroyed by the defenses of the body. If an insect receives a number of subminimal doses which, when totaled, exceed a M.I.D., it is reasonable to suppose that whether or not the insect falls victim to the disease depends upon the rapidity with which the fractional doses are received and upon whether this rate is greater than the rate at which the doses of the infectious agent are destroyed. If the defense mechanisms of the insect's body can destroy more organisms per unit of time than the amount of microorganisms received per the same unit of time, no infection takes place. If, however, the amount of microorganisms received by the host is distinctly greater over a period of time than the amount destroyed, infection is likely to result.

Methods of transmission and portals of entry have been discussed earlier in this chapter in the section on infections.

It is obvious that in an epizootic the susceptible insect hosts must be distributed spatially in such a manner that the disease agent can get from one host to another; otherwise the infection could not be maintained long enough for it to gain epizootic proportions. If for all practical purposes individual hosts are so widely separated that the infecting agent cannot travel from a diseased insect to a healthy one, no epizootic is possible. On the other hand, close and intimate contact between members of a population enhances the chances for an outbreak of disease. This idea is an expression of the principle that since contagious diseases among insects are density-dependent agencies, the density of the host population is of great importance in the manifestation of a naturally induced epizootic.

The Epizootic Wave

A typical epizootic usually shows a form of variation in time known as the "epizootic wave." The number of insects afflicted with a disease may

rise rather rapidly to a maximum or peak, then fall with varying degrees of rapidity back either to zero or to the normal, enzootic level. The curve representing this epizootic wave consists essentially of two parts: the ascending limb and the descending limb. Three types of curve can be described (Fig. 59): (1) with a longer descending than ascending limb; (2) symmetrical or nearly symmetrical, usually bell-shaped; (3) with a longer ascending than descending limb (Stallybrass, 1931).

The Preepizootic Phase. With respect to the relation between the infecting organism and its host, several things may take place during the

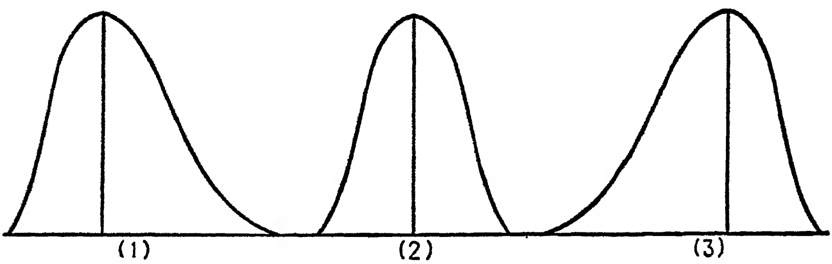

Fig. 59. The three types of curves that may describe an epizootic wave. 1. Curve of epizootic having a longer descending than ascending phase. 2. Symmetrical or nearly symmetrical curve of epizootic having equivalent or almost equivalent ascending and descending phases. 3. Curve of epizootic having a longer ascending than descending phase.

preepizootic phase, *i.e.*, the period just preceding the increased prevalance of the disease under consideration. For one thing the epizootic potential of the infecting organism may be rising as the result of an increase in virulence for the insect. It is well known, for example, that the virulence of certain entomogenous bacteria is enhanced in nature by the repeated passage of the organisms through successive hosts. On the other hand, the susceptibility of the host may be undergoing a change because of any of several intrinsic or environmental influences that may play upon it. During this phase there is also a greater degree of dissemination of the infecting agent, as well as an increasing rate of transmission from infected insects to healthy ones. There is probably an increased velocity of infection or a rising dosage of microorganisms received daily by the insects.

The density of the population during the preepizootic phase is also important since if only a few insects are present the infection is likely to die out before it has a chance to enter the epizootic phase. On the other hand, if a sufficiently large population exists, the preepizootic phase will end rather sharply, with a sudden rise in mortality introducing the epizootic phase.

The Epizootic Phase. Unfortunately an adequate amount of data pertaining to epizootics in insect population has been gathered only in a few isolated instances. Accordingly, very little actually is known concerning what transpires during this climactic phase of the epizootic wave. A rough guess as to what takes place may be gained from the information obtained by Topley (1926), Greenwood, Hill, Topley, and Wilson (1936), and others in the study of experimental epizootics of bacterial and virus infections among mice and other animals.

From the meager evidence at hand, it would appear that once an epizootic has begun it can be maintained indefinitely by the regular addition of equal numbers of animals. Instead of maintaining a regular level, however, the disease waxes and wanes, and a series of waves of mortality occurs; these waves vary in duration and amplitude in relation to the numbers of susceptible animals added daily. The wave length is shortest when many animals are added daily, and longer when smaller numbers are added. The intensity of the epizootic bears a direct relation to the rate of fresh exposures. When, however, extremely large numbers of animals are added, the wave form is less marked, and the epizootic may tend to die out.

Of particular interest is the fact that, when a fresh group of animals is added to the survivors of an epizootic, a new preepizootic phase results, followed by an epizootic wave in which many of the original survivors are killed off. In other words, at the end of the first epizootic wave a state of equilibrium exists between the parasite and its host, which is disrupted by the introduction of a group of susceptible newcomers.

Working with mice, Topley found that, if susceptible mice were continuously added to the infected population at a constant rate, the spread of infection continued indefinitely. Depending upon the rate of addition of fresh mice, there resulted either a constant and high enzootic of disease or a series of regularly recurring waves. Wave after wave could be produced until the original population had been entirely killed off. Whether or not a similar situation prevails in the case of epizootics among insect populations, it is difficult to say with any degree of certainty. It is entirely possible, however, that the two situations would be analogous.

At this point it is well to emphasize the fact that in any population there is a critical proportion of susceptibles and immunes. When there is a greater number of susceptibles than immunes, epizootic disease can develop; with a lesser number, it cannot. This critical point prevails at the peak of the epizootic wave and is called the "threshold density." Accordingly, the total number of cases in an epizootic will be twice the number of susceptibles in excess of this threshold density present at the beginning. Actually, in nature, the threshold density fluctuates and is

rarely the precise phenomenon just indicated. It is, in part, a function of dosage.

Postepizootic Phase. In the economy of nature it is characteristic of a population that suffers disaster that at least a few survivors always remain to perpetuate the species. So it is after an epizootic; and here the words of Kirby and Spence (1826), two early and famous entomologists who were among the first to recognize the significance of insect diseases and who wrote a chapter on this subject in their classic book, are of bibliographical interest: "The same Almighty Power which endowed them [insects] with so complex a structure, generally upholds them in health during their destined career, until they have fulfilled the purpose of their creation, when 'they die and return again to their dust.'"

Accordingly, the end of a single epizootic wave is characterized by the number and character of the survivors. The number of survivors varies with the epizootic, and in general two possible explanations of the varying proportions surviving after different epizootics may be offered.

In the first place, the original susceptibilities of the insects concerned may have been different, and for this reason a higher proportion of survivors actually represents a higher original resistance of the surviving insects.

The second explanation might be that the survivors have acquired an immunity to the infecting organism. To what extent this immunity factor is significant in epizootics among insects is difficult to judge on the basis of present information. In the case of experimental epizootics among small vertebrates it has been determined that the immunity of all survivors is at a higher level than that of any newcomers or the original animals. This immunity is rarely a complete immunity, however, and some investigators have concluded that a degree of immunity that may save individual animals, when living among equally resistant companions, is of very little protective value when they are surrounded by highly susceptible individuals of the same species.

The degree of dispersion of the survivors among fresh hosts is also a factor affecting the proportion of survivors. In general, substantial dispersion during the earlier phases of an epizootic tends to reduce the total mortality. When the epizootic wave has begun to rise, however, the effect of such dispersion on the total mortality is less.

In their experiments with mice, Greenwood *et al.* (1936) came to the conclusion that a disease will never normally die out, provided that the population is not reduced to such small numbers that the disease becomes extinct for this reason. The length of time that may pass before another epizootic wave develops depends, of course, on several factors, one of the most important of which is the rate of immigration by fresh hosts. When

the rate of immigration is low the mortality curve will consist of well-separated waves and quiet intervals. When the rate of immigration is high there will be minor fluctuations or a death rate with almost no fluctuations. Over a long period of time the average death rate is not highly correlated with the immigration rate, and there is a tendency toward a constant total population with a more or less constant death rate. Greenwood and his coworkers believe that this condition of equilibrium is fundamentally unstable, even though it is continued for long periods of time. When it is markedly disturbed, the system tends to pass through a period of violent fluctuations before equilibrium is once more established at the same, or some other, level. On the other hand, there are distinct types of fluctuation in the prevalence of disease which should be distinguished by the epizootiologist.

Variations in Disease Prevalence

As they concern the epidemiology of diseases occurring among human beings, Stallybrass (1931) considers the variations in the prevalence of disease under four headings. In general these same categories may be applied in the case of diseases among insects. They are as follows:

1. *Short-time irregular fluctuations* due to a variety of causes. Among the factors that may cause such fluctuations in the prevalence of insect diseases are included variations in temperature, humidity, nourishment of host (qualitative and quantitative), population densities, intercurrent infection and parasitism, and such man-made factors as the application of insecticides. These factors will be discussed further in the chapter on microbial methods of biological control (Chap. 14).

2. *Annual seasonal variations*, dependent more or less directly upon the seasonal meteorological changes, but also dependent to some extent upon the effect of seasonal changes in the behavior of the animals. It is a common thing to observe the prevalence of certain insect diseases at particular seasons of the year, depending upon the geographical location concerned. For example, some fungous diseases are noticed especially in the spring of the year, others during the fall, and some during the summer or the rainy winter. Epizootics of certain virus diseases occur during the late summer or fall and are relatively rare at other seasons of the year.

3. *Cyclic or intrinsic periodicities* of duration in months and years but not coinciding with the annual solar cycle. Some insect diseases appear to flare up in epizootic form every so many years. Whether or not these recurrences represent actual cyclic periodicity is not definitely known in most cases, but it is a likely possibility.

4. *Secular variations* in prevalence such as might be observed from one century to the next. Such variations have not been studied with regard

to insect diseases, although it is possible that such have occurred under man's observation, especially in the case of some of the diseases of the honeybee and the silkworm. At any rate, the inception of sanitary measures in the case of silkworm rearing has substantially altered the picture during the past century with regard to the diseases of this insect.

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CHAPTER 7

RESISTANCE AND IMMUNITY

During an epizootic of disease, certain members of the insect population do not succumb to the disease so readily as do others, or some may not succumb at all, and many of the survivors are less susceptible to the infection than they were prior to the outbreak of the disease. We say that these individuals are "resistant to the disease" or that they possess an "immunity" to the infecting agent. The present chapter will be concerned with the nature of this resistance or immunity and the role it plays in the economy of insect life.

It is convenient to consider the general subject of immunity on the basis of several rather distinct, although not altogether independent, categories:

I. Innate immunity or resistance
II. Acquired immunity
   1. Naturally acquired
      a. Active
      b. Passive (congenital)
   2. Artificially acquired
      a. Active
      b. Passive

Each of these categories will be treated in further detail at appropriate points in the chapter. Suffice it to say here that innate immunity is independent of previous contacts with the microbial parasite and is sometimes designated by the general term "resistance." Acquired immunity simply refers to an immunity that has been acquired sometime during the life of the animal. It may be naturally or artificially acquired, and this acquisition may come about through the active or passive participation of the host. The mechanisms by which acquired immunity operate are sometimes characterized as being either humoral or cellular in nature, depending on whether the factors concerned are associated with the serum or other body fluids of the host or with the physicochemical or more or less mechanical activity of certain cells, such as phagocytes, which attack the invading parasite.

INNATE IMMUNITY OR RESISTANCE

Sometimes the terms "innate immunity," "natural immunity," and "normal immunity," are used to include those innate or inherent

qualities of an animal's make-up which enable it to withstand or avoid invasion by certain microorganisms. The animal comes by this ability naturally. In most, if not all, cases of so-called "natural immunity," specific humoral antibodies play no important part. The "immunity" is instead the result of the role played by numerous other factors, largely mechanical and physiological in nature, which go to make up the animal's resistance toward infection.

Before taking up the mechanisms of resistance that come into play in the individual insect, let us first consider some of the broader, more general aspects of the phenomenon of resistance as it applies to species or to groups of insects.

Phylogenetic Resistance. Generally speaking, animals are not susceptible to microorganisms that cause diseases of plants, and vice versa. Thus there exists what may be spoken of as a "kingdom resistance" (or "kingdom immunity"). Similarly, within each of the kingdoms a general lack of susceptibility is exhibited by the members of each of the larger phylogenetic groups to the microorganisms affecting the members of the other groups. For example, the microorganisms causing diseases of fish are usually not pathogenic for mammals or for birds or for molluscs or for earthworms, and vice versa. In a few instances such cross infections occur in nature, but they are relatively few until one deals with animals all in the same minor taxonomic category (*e.g.*, genus) or in closely related categories.

As a group insects possess a relatively marked degree of natural resistance to most microorganisms pathogenic for vertebrates. And to a certain extent the converse is also true: many microorganisms having little or no pathogenicity for vertebrates are highly pathogenic for insects. The insusceptibility of insects to human pathogens has been demonstrated many times. On such insects as the larvae of the wax moth and the European corn borer, the tubercle bacillus (*Mycobacterium tuberculosis* (Schroeter)) has virtually no pathogenic or lethal effect. Similar insusceptibility is shown to such well-known human pathogens as *Pasteurella pestis* (L. & H.) of plague, *Corynebacterium diphtheriae* (Flügge) of diphtheria, *Mycobacterium leprae* (A.-H.) of leprosy, *Clostridium tetani* (Flügge) of tetanus, and certain streptococci. These bacteria, when administered to an insect by feeding or by injection, are either rapidly destroyed by the arthropod's defense mechanisms or are walled off in some part of the body and rendered innocuous.

Within the class Hexapoda, considerable resistance is exhibited between the members of one group to the diseases of another group. For example, the viruses responsible for polyhedroses infect insects predominantly in the orders Lepidoptera and Hymenoptera, and to a much lesser extent in the

Diptera. There are no well-authenticated instances of virus infections occurring naturally in members of any of the other orders. Furthermore, evidence at hand indicates that there is little cross susceptibility among species in the same order. For example, the silkworm is not very susceptible to the virus causing polyhedrosis in the gypsy moth, and vice versa. This species resistance is not confined to virus infections but also has been noted with certain protozoa, fungous, and bacterial infections. Sometimes most of the species in a single genus are susceptible to a particular infecting agent, but not those of a closely related genus. Similarly the members of the genera of one family might be susceptible to a disease while the members of a closely related family are typically resistant.

The possibility of differences in susceptibility being exhibited by members of infraspecific categories remains to be proved in most cases. Caucasian and Carniolan races of honeybees, *Apis mellifera* (Linn.), appear to be more resistant to *Bacillus alvei* Ches. & Chey., the cause of European foulbrood, than the common black bees. The Italian races appear to be more resistant than the Italian-black hybrid bees. To what extent such racial resistance prevails in other species of insects affected by other pathogenic agents is far from being adequately known. Certainly this information would seem to be of considerable economic value. Such proved to be the case when Pasteur was able to rear a stock of silkworms that were insusceptible to the microsporidian responsible for pebrine. He was able to do this by careful selection of individual silkworms immune to the disease, subsequently rearing them in pure strains. This discovery, as much as any other, saved the silk industry of France.

Age and Stage. Since the age of an insect is punctuated by various stages (egg, larval, nymphal, pupal, and adult), both age and stage must in many respects be considered together when resistance to infection is concerned. Larval and nymphal stages are, in turn, punctuated by molting periods or instars. The effect of the age or the instar or the stage of an insect on its resistance to infectious disease is extremely variable between species and groups of insects. Some insects are susceptible to a certain infectious agent only in one stage, while others are susceptible in all stages, and still others are resistant in all stages. Sometimes there is a direct relation between age and resistance, the young usually being more susceptible than older individuals. The increase in resistance coincident with the development of an animal to maturity has been designated by some authors as a "maturation immunity." The assumption is that this increasing resistance is associated with hormone production. No conclusive experimental proof of this hypothesis, however, has been provided in the case of insects. It has been clearly demonstrated that many

insects, particularly those having a complete metamorphosis, become resistant to certain types of infectious agents as they pass into the adult stage. This is particularly noticeable, for example, in the case of the various polyhedroses, which readily attack the larvae and even the pupae of certain lepidopterous and hymenopterous insects but do not materially affect the adults. Such is not always the case, however, and certain protozoan infections may flourish in either the immature or in the adult stages. In some cases the larvae acquire the infecting agent that produces a frank infection in the adult. On the other hand, certain diseases affect only the mature or adult stage and none of the immature stages, as is the case of nosema disease of the honeybee.

The relation of age to resistance to infection is, of course, influenced by many factors. It may depend rarely on the presence of true antibodies; more probably it depends on intrinsic qualities of the insect's tissues or metabolism or on the physiology or nature of the infecting agent.

Parenthetically, it might be mentioned here that some diseases exhibit a difference in sex incidence. For example, female hymenopterous parasites may become infected with the microsporidia of their hosts more frequently than do the male parasites. In nearly all such cases, it must be remembered that the observed sex differences may be explained on the basis of such factors as differences in activity (ovipositing in the above instance), relative numbers, risk of exposure, and the like.

Physiological Factors. The general physiological state of an insect exerts a profound influence on its resistance to diseases. As a rule resistance is greatest when the insect is functioning normally in every way. When this normal functioning becomes altered or is interfered with, the degree of resistance ordinarily possessed by the insect may be reduced. This deficient physiological state may be brought about by faulty nutrition, fatigue, various imbalances in metabolism, and the like. Sometimes a preexisting infection may predispose an insect to a secondary infection caused by an ordinarily harmless microorganism.

Unfortunately these factors in relation to resistance have had, in general, so little study as far as insects are concerned that we are left with only surmises and opinions based upon studies made in this connection on other forms of life. We can suppose, for example, that a deficiency in certain vitamins may result in a lowering of resistance. The same may be said concerning other essential food substances.

The effect of climatic factors, particularly temperature and humidity, on the well-being of insects is well known, but it is not clear how this affects the insect's resistance. Since some climatic factors may affect the infecting microorganism itself, it is difficult to differentiate between factors

affecting the host and those affecting the microorganisms. Relatively high temperatures and high humidities appear to favor the development of most infections in insects, but how much of this is due to the lowering of the host's resistance and how much to the enhanced growth of the microorganism has not been determined generally. It is possible, however, that resistance may vary with the season, temperature, humidity, and similar factors. More investigation covering these points is needed.

Sometimes it is difficult to differentiate clearly between physiological defense mechanisms and those that are purely mechanical. For example, when a considerable number of *Culex pipiens* Linn. are fed on avian blood containing thousands of infective forms of *Plasmodium cathemerium* Hart. or *Plasmodium relictum* G. & F., some of them become heavily infected, others lightly, and some not at all. These noninfected individuals remain so even after repeated infective feedings. By a selection of progenies from susceptible and insusceptible females, Huff (1940) was able to maintain stocks with increasing and decreasing degrees of susceptibility. He noticed that this character of insusceptibility behaved as a Mendelian dominant and concluded that the immunity barrier in this case appeared to be the intestinal wall. Perhaps both mechanical and physiological attributes of the host play a role in "immunity" mechanisms such as this.

External Defenses of the Insect. The body cavity of an insect is in actuality a closed system with respect to the outside environment from which it is separated by the integument and the intestinal epithelium. These structures constitute natural barriers to infection and have been designated as the "first lines of defense."

The intact integument, or exoskeleton, is a particularly effective barrier to invading microorganisms; few bacteria, viruses, or protozoa are capable of penetrating it. Only fungi and such organisms as nematodes and parasitic insects can penetrate this armor. It serves essentially as a mechanical barrier, since it consists of a rather thick, impermeable, nonliving, cuticular wall.

Although not so impervious as the integument, the intestinal epithelium (covered by chitin in the foregut and hindgut but not in the midgut) also forms an effective barrier to prevent many microorganisms from entering the body cavity. The epithelial lining in the midgut is the portal of entry for many virulent bacteria, viruses, and protozoa, but the number and variety of microorganisms that are kept out are vastly greater than the number that are able to invade this barrier. In fact, it is cause for wonder that the thin layer of epithelium in the midgut of insects is able to keep out of the body cavity the tremendous number of microorganisms that frequently fill the lumen. In many insects the peritrophic membrane aids substantially in maintaining the protective devices of the gut wall.

CELLULAR IMMUNITY

Ever since Metchnikoff's discovery, in 1883, that certain wandering cells in the body cavity of a water-flea (*Daphnia*) were capable of engulfing and destroying the spores of an infecting yeast, the importance of cellular activity in the protection of animals against infection has been realized. This type of protection is frequently spoken of as "cellular immunity."

Metchnikoff was particularly concerned with the action of certain wandering cells of larval starfish which, by amoeboid motion, were capable of ingesting particles of carmine and other substances injected into the body cavity. Similar cells, either fixed or moving, are common to all animals, including insects. Metchnikoff called these cells "phagocytes," from the Greek meaning "cells which devour," and to the process he gave the name "phagocytosis." Of a bacterium or particle engulfed by such a cell, we say it is "phagocytosed."

In the majority of animals, the phagocytes most easily demonstrated are usually found in the blood stream. So it is with insects. Because not all hemocytes or blood cells are equally phagocytic, it is important for us to have some idea as to the cellular make-up of insect blood. In fact, a consideration of the entire circulatory system is apropos at this point, since it will also apply to our discussion of humoral immunity a few pages later.

The Circulatory System. The circulatory system of insects is not enclosed in a system of veins and arteries as it is in higher animals. Instead the blood fluid (hemolymph or plasma) and the blood cells (hemocytes) circulate freely throughout the body cavity (hemocoele) of the insect, bathing all the organs and tissues directly. In most insects the hemocoele is divided into sinuses by fibromuscular septa, or diaphragms. Dorsal to the alimentary tract is located the dorsal vessel, the principal organ of the circulatory system. The posterior part of this structure is differentiated into the heart; the anterior part is the aorta. The dorsal vessel usually appears simply as a narrow, continuous or chambered, tubelike structure, the sides of which are perforated with small valvular openings called "ostia." The blood is kept in motion throughout the hemocoele by contractile waves or pulsations of the heart or, in some cases, of the entire dorsal vessel. Pulsatile organs may also occur in other parts of the body to assist in the thorough distribution of the blood.

Unlike vertebrates, insects do not have erythrocytes or red-blood cells, and, except for chironomids, they do not have oxygen-carrying compounds such as hemoglobin in their blood. Hence the blood plays a very small part in the insect's respiration, a function carried on largely by the tracheal

system, which takes care of the gaseous exchanges. The main function of the blood is to convey nutrient substances to the tissues and to carry waste materials to the excretory organs.

In many insects, the hemolymph or plasma also has the property of clotting. Yeager and Knight (1933) have divided insects into three groups on the basis of the clotting properties of their blood: (1) species in which the blood does not clot, (2) species in which the clot is formed by agglutination of the hemocytes, and (3) species in which the clot is formed by coagulation of the plasma.

The hemolymph is a more or less viscid liquid that may be clear or tinged with green, yellow, orange, or brown pigment. The color is generally characteristic of the species and has no relation to food or to geographical location. In some lepidopterous larvae and pupae the color differs with the sex, usually being green in female caterpillars and yellow or colorless in males. Long lists of substances found in the hemolymph of insects have been published (*e.g.*, see Muttkowski, 1924), but in many insects four main constituents have been distinguished. These are fibrin, lutein, uranidin, and hemoxanthin. The amount of each probably varies considerably with the species concerned.

The Blood Cells. The blood cells, or hemocytes, of insects are difficult to place in any one particular type of arrangement. A number of classifications have been proposed, but no one of them has been found to be applicable to all species of insects. In the first place, there is so much variation in the hemocyte picture of different species of insects that a general classification, if it is to include all types, must be made so broad as to be of little practical value. Furthermore, unlike mammalian blood, that of insects contains the various transitional and developmental stages as well as the mature hemocytes.

It is not our purpose here to discuss the numerous classifications of hemocytes that have been set forth by different authors. The reader interested in this phase of the subject may consult such publications as those by Hollande (1911), Muttkowski (1924), Paillot (1933), Cameron (1934), Rooseboom (1937), Yeager (1945), and Millara (1947). Yeager's excellent study of the blood picture of the southern armyworm, *Prodenia eridania* (Cram.), enabled him to set up a comprehensive classification of all the hemocyte variations occurring in this insect. Although a detailed breakdown is certainly desirable as far as the gaining of an accurate knowledge of insect hematology is concerned, perhaps for the present it is more desirable for us to choose a simpler classification so as to be able to apply it to the accumulated information we have on phagocytosis. Even the simplified classifications that have been proposed are not entirely satisfactory, and we shall therefore have to select one on a purely arbitrary

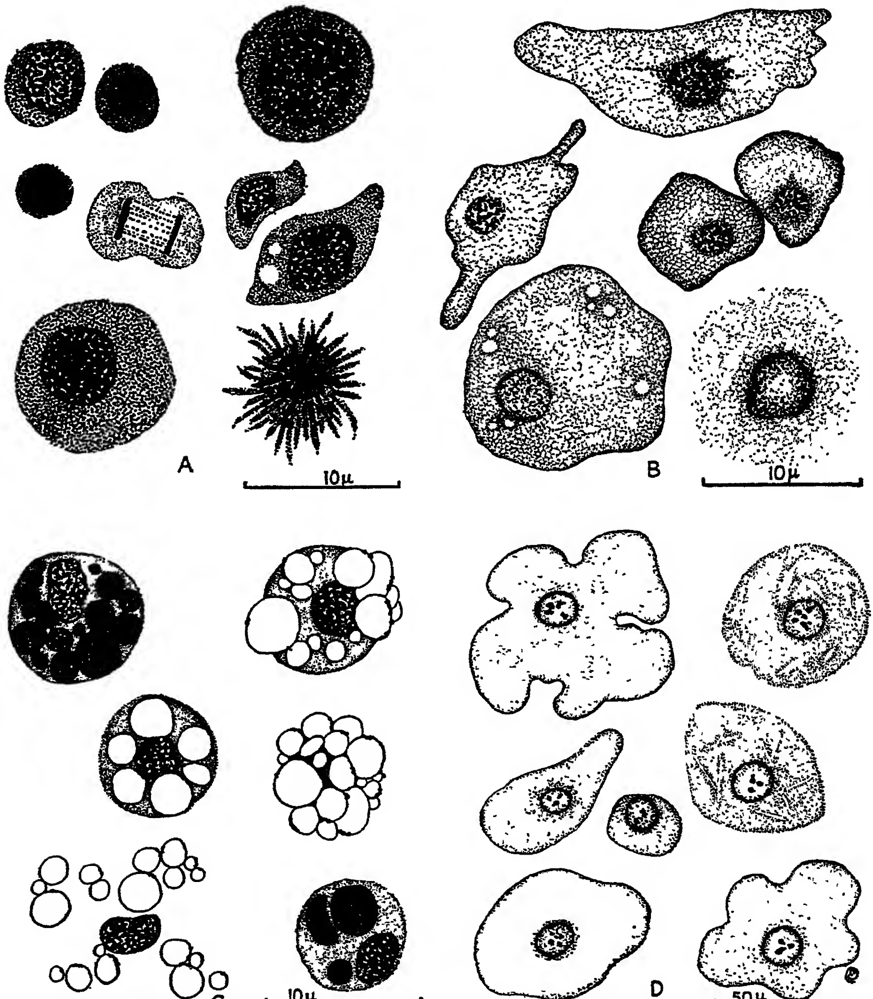

Fig. 60. The principal types of hemocytes in insects. The more general forms of each type are represented. Diagrammatic. *A*. Lymphocytes. *B*. Leucocytes. *C*. Spherule cells. *D*. Oenocytes or oenocytoids, as depicted by Wigglesworth and others.

basis. Let us consider the one used by Cameron (1934) in his studies on inflammation in caterpillars of Lepidoptera, particularly those of the wax moth *Galleria mellonella* (Linn.).

Cameron distinguishes four principal groups of hemocytes, as follows:

1. *Lymphocytes*. Vary in size from 4 to 12 microns, occasionally up to 20 microns, in diameter. Are characterized by a deeply staining, round or oval nucleus,

and scanty well-stained cytoplasm. They are round, oval, irregular, or spindle-shaped. Vacuoles may be present. Mitoses are not uncommon. Show active amoeboid movements. Comparable to the proleucocytes, amoebocytes, and macronucleocytes of other authors.

2. *Leucocytes.* Generally much larger than lymphocytes, varying in diameter from 10 to 25 microns, but smaller and larger forms occur. They have a small, oval or round, well-stained nucleus centrally or peripherally placed. Mitoses are seldom seen. The amount of cytoplasm is relatively great, and it is faintly staining and usually homogeneous. Not infrequently small unstained vacuoles occupy a considerable portion of the cytoplasm. The cells are actively amoeboid. They may show well-defined processes, or they may be spindle-shaped with long-drawn-out extremities. Comparable to the large amoebocytes, micronucleocytes, and phagocytes of other authors.

3. *Spherule Cells.* About the same size as the lymphocytes and are characterized by the large number of coarse spherules which practically fill up the entire cell, so that it is difficult to make out the nucleus. Properly stained the spherules are acidophilic. They do not stain with Sudan III or with osmic acid, and give typical protein reactions. The spherules are eventually discharged from the cell and not infrequently a spherule cell may be seen in a state of disintegration. More frequently only a few are discharged at a time, the cytoplasm then showing a vacuolated appearance. The liberated spherules apparently go into solution in the hemolymph. The discharge of the spherules is well marked in the larva as it goes into pupation. Spherule cells seldom show any movements.

4. *Oenocytes.* Very large cells with homogeneous, deeply acidophilic or amphophilic cytoplasm, and very small well-stained nuclei. Fine linear markings may appear in the cytoplasm. Some authors designated cells of this description which occur free in the blood as *oenocytoids* to differentiate them from the oenocytes (Gr. "wine-colored cells") associated with the fat body or attached to the tracheae. They also have been called *cerodecytes.* Cameron believes that such differentiation is unnecessary, but other authors (*e.g.*, Wigglesworth, 1933) have pointed out that in some insects, at least, they have different origins.

Differential counts to determine the relative proportions of the various types of blood cells have been made on very few species of insects. For this reason no generalized statement concerning differential counts in insects can be made. Cameron (1934), as well as Metalnikov (1927), made counts on the blood of *Galleria mellonella* (Linn.) and obtained figures approximately as follows: lymphocytes, 38 to 45 per cent; leucocytes, 50 to 57 per cent; spherule cells, 3 to 4 per cent; oenocytoids, less than 1 per cent. Toward the end of the larval stage, the leucocytes are usually more numerous, the proportion of lymphocytes being decreased. After a hemorrhage, such as might be caused by the withdrawal of blood for examination, the proportion of lymphocytes may increase while that of the leucocytes becomes relatively decreased. Somewhat wider percentage ranges have been noted in other insects. Thus in *Pyrausta nubilalis*

(Hbn.) the percentage of lymphocytes runs between 27 and 45 per cent; leucocytes, from 30 to 69 per cent; and spherule cells, from 2 to 8 per cent. In most of the cases so far studied, bacterial infections in insects give rise to an increase in the number of lymphocytes and a decrease in the number of leucocytes. This alteration in numbers usually occurs very rapidly (30 to 60 minutes) after introduction of the bacteria.

The total number of blood cells in insects is extremely variable with the species (as well as with the higher categories), and values have been reported as low as 200 or 300 cells in the entire insect to at least as high as 275,000 cells per cubic millimeter. The average probably ranges somewhere between 10,000 and 30,000 cells per cubic millimeter.

Phagocytic Cells of Insects. Most insects appear to have at least two, and probably three, different types of phagocytic cells in their bodies: (1) certain of the blood cells; (2) the pericardial cells; and, according to some authors, (3) certain cells of the fat body.

Of the blood cells or hemocytes, the leucocytes generally assume the most active phagocytic role. They readily engulf bacteria and other foreign particles introduced into the blood. The lymphocytes are next in importance as phagocytes, but they generally are not so active as the leucocytes. Occasionally the spherule cells are phagocytic, but the oenocytoids are not. Sometimes several types of cells function together to surround a foreign body, forming what is known as a "giant cell." The hemocytes may form giant cells by fusing into multinucleate masses. Giant cells may also form by the hypertrophy of single cells, usually lymphocytes, and they are then sometimes known as "teratocytes."

The pericardial cells, like the hemocytes, are of mesodermic origin. They may be found in different arrangements, but they are not migrating cells and usually remain in a rather fixed position. They nearly always lie in the region of the heart (see Fig. 61), a common location being along the diaphragm and connective tissues supporting the heart. Some authorities consider the pericardial cells as belonging to the same system as certain "excretory" cells found throughout the insect body and designate the whole as "nephrocytes," from the belief that they may play some intermediate role in the segregation and storage of waste products. In many respects these cells, including the pericardial cells, may be likened to the reticulo-endothelial system of vertebrates. Their ability to absorb colloidal particles from the blood has been demonstrated beyond question. The pericardial cells are very large and usually contain more than one nucleus, and the cytoplasm, especially of the innermost cells, may be vacuolated. Sometimes they may contain red, brown, yellow, or green pigments.

As Cameron (1934) has pointed out, scattered throughout the lobules

of the fat body are small collections of cells, resembling lymphocytes, suggestive of a hemopoietic center in the mammalian fetus. Cameron

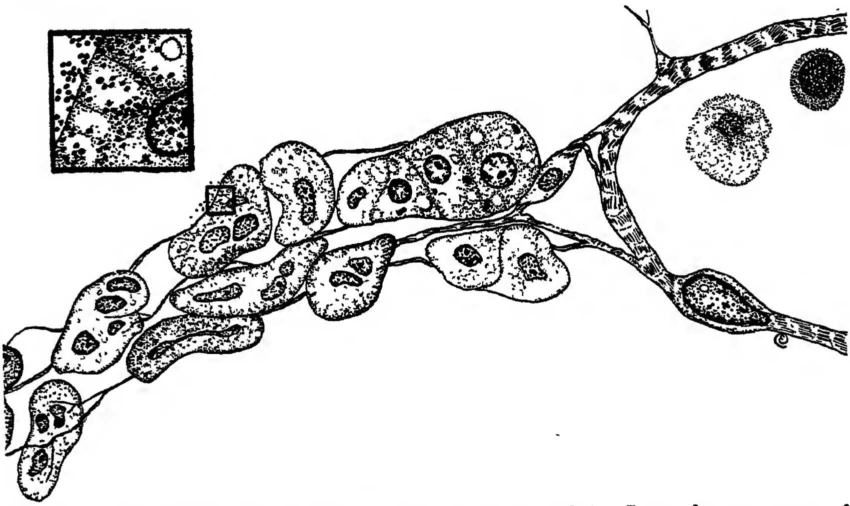

Fig. 61. Pericardial cells, extending from the heart (right). Inset shows process of phagocytosis.

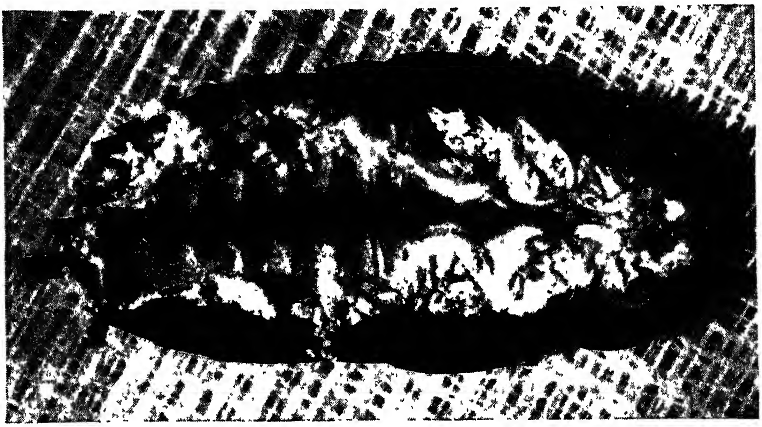

Fig. 62. Dissected cockroach, *Periplaneta americana* (Linn.), 24 hours after injection of trypan blue solution, showing the deeply stained pericardial cells distributed among the alary muscle fibers along the heart. (*Courtesy of J. Franklin Yeager; see Yeager et al., 1942.*)

believes that these groups of cells are, in part, responsible for the production of free blood cells. These, and certain other cells in the fat body, have been considered by some to be part of the nephrocyte system mentioned in the last paragraph.

Phagocytosis in Insects. If one introduces into the body cavity of an insect a suspension of foreign particles, almost at once the freely mobile phagocytic cells of the blood begin to engulf the particles. Within a short time the fixed phagocytic cells of the fat body and the pericardial system may also exert their phagocytic action. Within a few hours or days, depending on the insect as well as on the nature of the introduced material most of the particles are taken in and digested or disposed of through the excretory system. When the foreign particle is a bacterium, it is of special importance to the life of the insect whether or not the phagocytes are able to engulf and destroy sufficient numbers of the organisms to save the life of the animal. In many cases the protection afforded the insect by virtue of its phagocytic cells is considerable and makes up an important part of the insect's defenses against infection.

Most of our information on the role of the phagocytes in cellular immunity is dependent upon the works of Hollande, Paillot, Metalnikov and his colleagues, and Cameron.

Whether the foreign particles introduced into the hemocoele of an insect are of animate or inanimate nature does not seem to alter substantially the type of phagocytic action that ensues. Materials such as colloidal iron, India ink, or carmine are readily phagocytosed by the leucocytes and lymphocytes of the blood, and by the phagocytic cells in the fat body of insects such as the larva of the wax moth. Although India ink is not taken up by the pericardial cells, colloidal iron and carmine are phagocytosed by these cells. This was the observation of Cameron, who also noticed the occurrence of a rapid increase in the proportion of lymphocytes within 2 to 4 hours after injection of a foreign material, although this passes off in 1 or 2 days. Large particles or clumps of particles are quickly surrounded by leucocytes and lymphocytes. Within 24 hours or so, encapsulation with the formation of a nodule occurs. This nodule persists throughout metamorphosis and can usually be found unchanged in the adult insect. Tissue cells and heterologous blood cells are removed partly by autolysis, partly by encapsulation, and partly by phagocytosis, although the latter is not a marked feature.

When bacteria are introduced or find their way into the body cavity, they may be phagocytosed in a manner essentially the same as that by which inert particles are engulfed. Some species of bacteria are taken up very rapidly, while others are phagocytosed in insignificant numbers only. Frequently the phagocytosed bacteria are surrounded by vacuoles, a characteristic usually not seen with engulfed inanimate particles. Several workers have observed the formation of a brownish-black pigment in many of the phagocytes in close association with the ingested bacteria or inert particles. This is thought to represent some physiological or me-

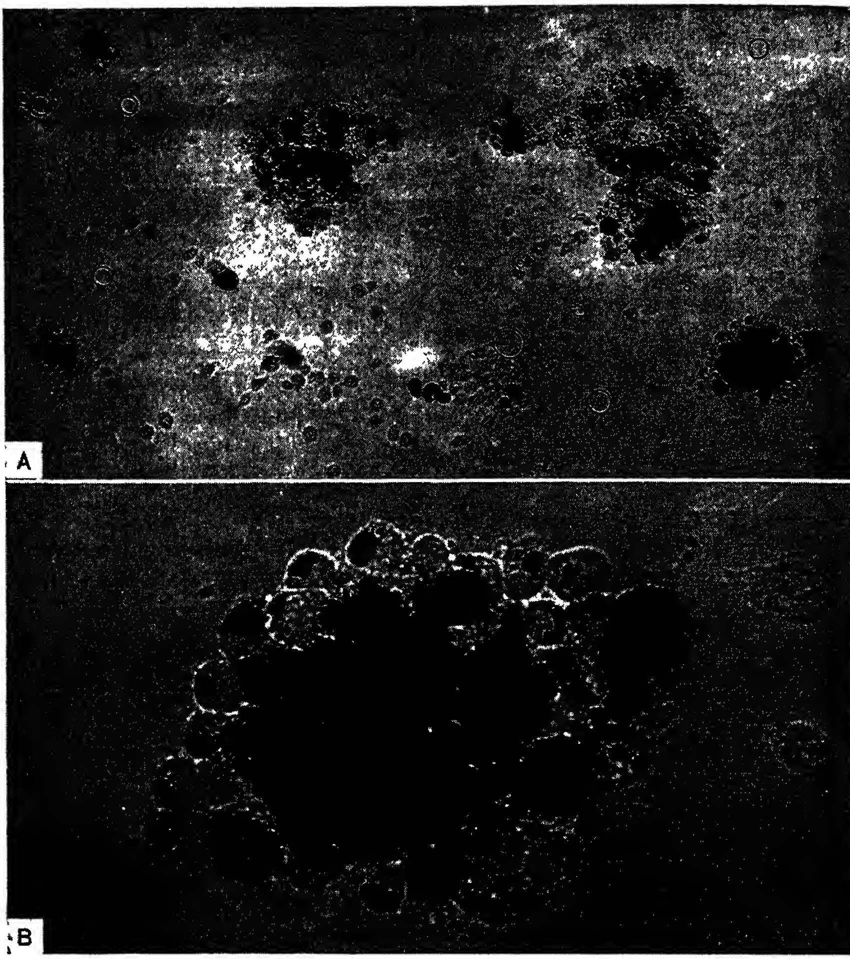

Fig. 63. Phagocytosis of Chinese ink particles by hemocytes of the American cockroach, *Periplaneta americana* (Linn.). *A*. Blood or hemolymph from a roach 24 hours after injection of ink suspension. A few cells contain no ink, some a small amount of ink, and some are heavily loaded. The plasma is almost free of ink particles. *B*. Clump of agglutinated hemocytes (seen at extreme right end of *A*) at a higher magnification. Some of the ink massed in the center of the agglutinated hemocytes may be encapsulated, but most of it is phagocytosed. (*Courtesy of J. Franklin Yeager; see Yeager et al., 1942.*)

tabolic modification of cell function and structure. In some insects, granules form in the phagocytes after phagocytosis has occurred. Sometimes these granules are eosinophilic, but their true significance is not known.

The phenomenon of phagocytosis of bacteria in insects has been studied with two different groups: those bacteria pathogenic for insects or normally found in association with them, and those bacteria entirely foreign to insects and in many cases pathogenic for vertebrates. If the bacteria, in the first of these groups, is highly pathogenic for the insect, infection and death are likely to ensue without much, if any, phagocytosis taking place. With bacteria of lesser virulence, more of them may be phagocytosed by the blood cells. Since most of the bacteria pathogenic for higher animals are of slight or little pathogenicity for insects, these forms are usually readily engulfed by the phagocytes. Nevertheless the phagocytes play an important role in protecting an insect against the ravages of virulent invading microorganisms. That they are not so important in this regard as was at first supposed now seems clear since there is ample proof that humoral immunity is of paramount importance in defending the body against disease agents.

Several species of insects have been studied with regard to the ability of their hemocytes to phagocytose bacteria, but two examples may suffice to explain the general types of reactions involved: the larvae of the European corn borer, *Pyrausta nubilalis* (Hbn.), and of the wax moth, *Galleria mellonella* (Linn.). Working with the first of these species, Metalnikov and Chorine (1929) found some bacteria (*e.g., Mycobacterium tuberculosis* (Schroeter), staphylococci, and micrococci) to be phagocytosed very rapidly—the leucocytes being most active in this regard with the lymphocytes next in importance. Little or no phagocytosis was provoked by those bacteria most virulent for the larvae, such as *Bacillus thuringiensis* Berl., *Bacillus canadensis* (Chor.), *Bacillus galleriae* No. 2 (M. & C.), *Bacterium ellingeri* (M. & C.), and *Vibrio leonardii* M. & C. With some bacteria (*e.g., Bacillus subtilis* Cohn) phagocytosis occurs only during the first stages of a mortal infection and then diminishes, until finally the bacteria multiply freely in the septicemic blood. With still other bacteria (*e.g., Proteus vulgaris* OX-19), no phagocytosis takes place at first, but later, 15 to 20 hours after infection, the lymphocytes may be found to have phagocytosed some of the bacteria. In the meantime, however, the bacteria have multiplied to such an extent, and the phagocytic reaction has occurred so late, that the insect cannot ward off the infection and dies a few hours later. Thus Metalnikov and Chorine observed the following types of phagocytosis to occur in the corn borer, depending upon the type of infecting bacterium: (1) complete or almost complete absence of phagocytosis; (2) phagocytosis at the beginning of the infection but gradually diminishing; (3) little or no phagocytosis at first, but gradually developing as the infection proceeds; (4) marked phagocytosis from the beginning to the end of the infection.

Working with the larvae of the wax moth and other insects, Cameron (1934) noticed a similar variation in the types of phagocytosis elicited by different bacteria. Thus, when inoculated with the living bacteria, the larvae may respond with any of the following reactions: (1) active phagocytosis and complete destruction of the bacteria (*Diplococcus pneumoniae* Weich., *Micrococcus* [*Staphylococcus*] *pyogenes* var. *aureus*

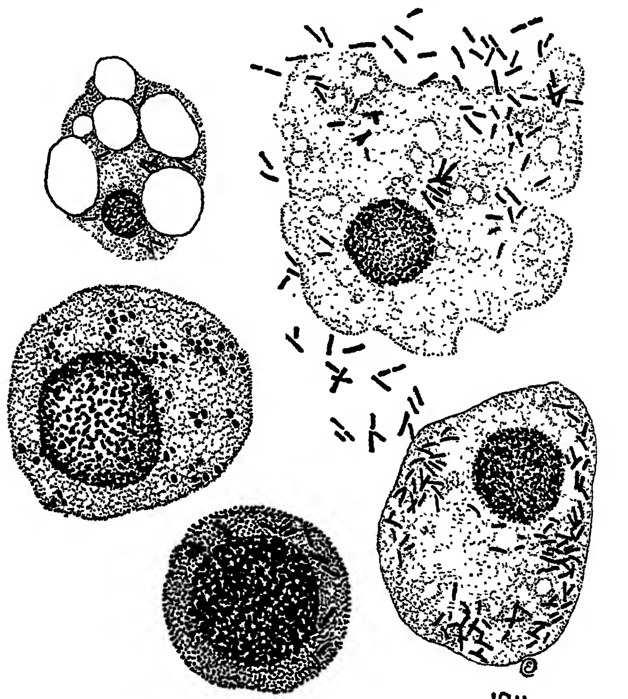

Fig. 64. Ceitain of the phagocytic hemocytes showing phagocytosed bacteria. Semi-diagrammatic.

(Ros.), *Hemophilus influenzae* L. & N.); (2) active phagocytosis, but growth of the bacteria, and rapid death of the larvae (*Proteus vulgaris* Haus., *Bacillus mycoides* Flügge); (3) active phagocytosis with survival of some organisms and their subsequent active growth, the larvae dying after several days (*Micrococcus* [*Staphylococcus*] *pyogenes* var. *albus* (Ros.), *Clostridium perfringens* (V. & Z.)); and (4) slight phagocytosis but destruction of bacteria, presumably by other immunity principles (*Salmonella typhosa* (Zopf), *Shigella dysenteriae* (Shiga), *Vibrio comma* (Schroeter)). Cameron found that acid-fast bacteria (*Mycobacterium tuberculosis* (Schroeter) *Mycobacterium smegmatis* (Trev.) [*M. lacticola* L. & N.]), although rapidly phagocytosed by the free and fixed phagocytes, survive

unchanged without exerting any injurious action on the larvae. They appear to be segregated chiefly in the pericardial cells, where they survive throughout metamorphosis, and can be isolated in a living virulent state from the adult moth.

The extrinsic conditions that tend to promote or to diminish phagocytosis have not been well studied. Marked variations in temperature cause corresponding variations in the speed at which the phagocytic reaction takes place. The beginning of phagocytosis is usually retarded at temperatures as low as 18 to 15°C. At temperatures of 10 to 12°C. it requires 1 or 2 hours for phagocytosis to begin. Below 10°C. practically no phagocytic activity occurs in most insects. Rather vague and general observations have indicated that the age and physical condition of the insect also affect the action of the phagocytes. Although the production of opsonins has not been demonstrated in the blood of insects, it is probable that such an antibody may be present. The phagocytes of naturally or artificially immunized insects usually exhibit an increased rate of phagocytosis from those of normal unimmunized individuals.

Unless the phagocyte is destroyed, the final phase of the phagocytic reaction is the intracellular digestion of the engulfed bacteria in the protoplasm of the phagocyte. Such dissolution of bacteria actually has been observed by microscopic examination. In undergoing this process the bacteria may take on a brown pigmentation before being finally dissolved. In other cases the organisms become swollen and are then gradually absorbed. Sometimes they first break up into granules which are then rapidly digested by the cell. When large numbers of bacteria are engulfed by a single phagocyte, this cell may become filled to such a point that it cannot effectively digest the organism and may itself be destroyed. Destruction of the phagocytes may also come about through the action of toxic substances secreted by the bacteria. Occasionally the phagocytes may be seen to become greatly hypertrophied with large vacuoles being formed in the cytoplasm. In such cases the phagocytes are destroyed, and the insect soon dies.

Segregation and Giant Cells. If a bacterium such as *Mycobacterium tuberculosis* (Schroeter) is injected into the body cavity of a wax-moth caterpillar or into that of a corn-borer larva, the phagocytes may unite in masses to encapsulate and segregate the foreign organisms. Such masses have been called "giant cells," "phagocytic complexes," "plasmodia," or "nodules" and have been observed to form in response to a variety of irritants, including inert particles. They were probably first observed in Orthoptera forming around encysted gregarines (protozoan parasites) and nematodes, and they have since been seen forming around such things as a distome fluke in a beetle, around a sporozoan in a mealybug,

around a coccidian in a meal-moth larva, and around certain bacteria in several different insects.

The giant cell or nodule is usually formed by leucocytes and lymphocytes which arrange themselves in concentric layers about such masses or aggregates as those made by tubercle bacilli so as to form a spherical or oval body. Brownish-black pigment appears at the center of the nodule and in the cells in immediate contact with the foreign material. After several days, according to Cameron (1934), fibrils appear at the periphery of the encapsulating cells, and eventually what resembles a fibrous tissue

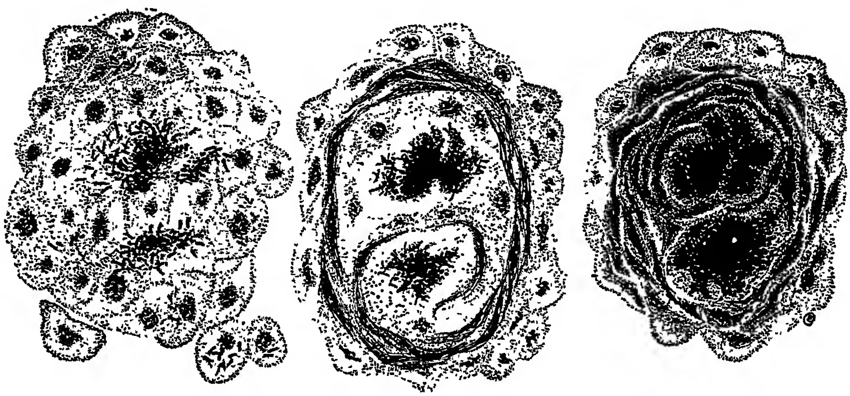

Fig. 65. Development of a giant cell, or nodule, as it occurs in the body cavity of such insects as the wax-moth caterpillar and the larva of the corn borer following the inoculation of a microorganism, *e.g.*, the tubercle bacillus. (See text.)

wall surrounds the nodule. The fibrils do not, however, give the usual reactions for collagen.

Metalnikov and Chorine (1929) describe the formation of these giant cells in corn-borer larvae inoculated with a large dose of tubercle bacilli somewhat as follows: The fusion of the phagocytes commences immediately after the bacteria are injected into the insect. Three to four hours later, large numbers of "plasmodia" filled with bacteria may be seen in the blood. They may be demonstrated in smears and especially in microtome sections. These plasmodia gradually become more compact, digest the bacteria, and transform them into a brown pigment. One or two days after the injection, a number of lymphocytes and leucocytes assemble about the surface of the giant cells in concentric layers, often taking the shape of long filaments. In this manner compact capsules or nodules are formed, within which the bacteria have been imprisoned and rendered harmless. Two things are thus accomplished: large numbers of bacteria are concentrated at one place, and there is an intensification of the intracellular digestion of the engulfed organism.

Similar phagocytic complexes have been observed in the case of infections caused by certain fungi, *e.g.*, *Sorosporella uvella* (Kr.). According to Speare (1920), the vegetative cells of this fungus are frequently seen within a mass of phagocytes which are arranged in irregularly concentric layers. Sometimes compound complexes are formed, in which there are two or three foci, or centers of attraction, the whole being surrounded by a common envelope composed of several layers of leucocytes. These complexes occur throughout the body cavity, though more commonly near the dorsal vessel or the tracheae of the insect host, usually a noctuid. Certain protozoa (*e.g.*, coccidia) elicit similar formations.

The ultimate fate of the nodule or giant cell formed within the body cavity of the insect varies somewhat depending upon its size, the nature of the contents, and the species of insect. Usually it undergoes very little change once it is completely formed. It may persist throughout the life of the insect, including the period of metamorphosis from larva to adult.

Formations analogous to abscesses in higher animals have been described in insects as the result of giant-cell formation. The disintegrating bacteria surrounded by phagocytes appear as pigmented spots immediately under the skin of the larva. Gradually the epidermis and cuticle over these spots become pigmented, and they finally burst, liberating the contents of the "abscess." After this occurs a new epidermis is formed underneath.

The part played by the pericardial cells in the segregation of living as well as inert particles has already been mentioned. Cameron (1934), in particular, has called attention to the importance of these cells in the defense mechanisms of insects. Bacteria may be phagocytosed by these cells and segregated therein in considerable numbers. Cameron maintains that at times the bacteria adapt themselves to existing conditions in the insect and establish a kind of symbiosis or mutualism with the fixed phagocytes of the pericardial system and the fat body, becoming segregated in these cells. Such, for example, may occur with certain of the coliform bacteria, which may be rapidly dealt with by the larva without much evidence of phagocytosis by the lymphocytes and leucocytes. Humoral mechanisms of defense may also play a role in cases such as this.

ACQUIRED IMMUNITY

An immunity that has been gained by an animal during its lifetime is said to be an "acquired immunity" and is to be distinguished from so-called "normal" or "natural immunity." An acquired immunity generally results from the activity of true antibodies, whereas innate immunity or resistance, in the strict sense, is not dependent upon the presence of antibodies. Such normally operating factors as phagocytosis are usually

enhanced in their activity and become more efficient defense mechanisms whenever an insect acquires immunity to a particular microorganism. Of greater importance, however, are the humoral responses which occur in most instances of acquired immunity.

The immunity of which we speak may be acquired naturally as the result of an attack by an infectious agent (naturally acquired immunity), or artificially when infectious agents or vaccines are inoculated into the insect (artificially acquired immunity). Each of these two types of acquired immunity may in turn be active or passive in character. An *active immunity* is simply an immunity in the production of which the host has had active or direct participation. Along with the production of antibodies by the host itself, there is usually an accompanying increased cellular reactivity and a general increase in resistance to the microorganism concerned. A *passive immunity* accrues to a host that is the recipient of antibodies formed in the body of another animal of the same or different species. It involves no active generation of protective substances by the immunized insect. In other words, the antibodies may be actively produced in one insect and then, by means of this insect's hemolymph, transferred to the body cavity of another insect, which in turn is said to possess a "passive immunity."

Antigens and Antibodies. Substances that are introduced into the blood or tissues of an animal and stimulate the production of antibodies are called "antigens." Thus microorganisms or infectious agents referred to in the preceding paragraph are antigens. Conversely, the substances produced in the body of an animal in response to the introduced antigen are called "antibodies." The one is defined in terms of the other.

It is generally considered that only foreign proteins may serve as antigens. This, of course, includes all bacteria, viruses, and other microorganisms, as well as the protein from any other species of animal or plant. There is some evidence that certain lipoid or carbohydrate substances may also stimulate the production of antibodies, but this property is by far the most common among the proteins, especially those having large complex molecules.

The antibodies produced by the introduction of any particular antigen are usually specific for that antigen and will react (either in vivo or in vitro) only with this or closely related antigens. Each antigen stimulates the production of a corresponding antibody. The exact chemical nature of antibodies is inadequately known. It is known that they are a part of the serum proteins called the "globulin fraction," and it appears clear that they are modified serum globulin. The chemical and physical properties of antibodies are therefore those of globulin.

The antibody content of the blood serum or hemolymph may be demon-

strated by various types of activity. Although it was at first believed that each activity was due to a separate substance, each of which was given a name of its own, present indications are that only one or two really different kinds of antibodies exist. What have appeared to be many different antibodies are actually only different types of reactions exhibited by the same substance. Nevertheless it is still convenient to refer to each of these activities separately and to use the distinctive names given to them.

Types of Antibody Activity in Insects. Not all the various kinds of antibody activity have been conclusively demonstrated in insects or with insect hemolymph, but some definitely have. With more painstaking investigation it is possible that all types of humoral activity now known in vertebrates will be recognized in insects.

Lysins are antibodies that act upon foreign cells, bringing about their dissolution or lysis. Bacteriolysins kill and dissolve bacteria. They are the most easily demonstrated antibodies found in the blood of insects. In many insects they appear to occur naturally, but this may represent previous specific or nonspecific antigenic contact. They may also be produced artificially by vaccination or by sublethal infection. On the basis of meager data, the bacteriolysins of insects appear to have characteristics slightly different from those of vertebrates. For example, unlike the bacteriolysins of vertebrates, those of insects cannot be separated into two portions by heating. Furthermore the bacteriolysins of insects are not entirely inactivated by temperatures up to 75°C. This has been demonstrated with the blood of the cutworm *Euxoa segetum* Schiff., which does not lose its bacteriolytic power against *Bacterium melolonthae nonliquefaciens* Pail. when heated at 70 to 72°C., although it may decrease slightly at the latter temperature and up to 75°C., after which it is destroyed (Paillot, 1933). In fact, Zernoff (1931) reports that, if the immune hemolymph of larvae of the wax moth and European corn borer is diluted in physiological saline, the bacteriolysins are not destroyed in 20 minutes at temperatures as high as 115°C.

Other properties of insect bacteriolysins are only inadequately known. Chloroform gradually destroys their activity. They may be preserved for several days in vitro. Apparently they are not highly specific, since they can sometimes be produced against one species of bacterium by the injection of another unrelated species. They have even been produced by foreign albuminoid substances. In most of these instances, however, the action of the bacteriolysins is weaker than is that of the specific lysins. In any case, the quantity of material injected into the insect is of only secondary importance in determining the strength of the bacteriolysin produced.

Bacteriolysins appear to be responsible for much of the immunity

exhibited by certain insects. Some insects appear to lose their immunity to certain bacteria as soon as the immune blood loses its bacteriolytic properties. In other cases, whereas the bacteriolysins disappear about 3 days after immunization, the immunity of the insect against the agent concerned maintains itself for several days longer. Bacteriolysins can usually be detected in the hemolymph of the insect in from 4 to 6 hours after immunization. Apparently not all insects produce bacteriolysins with equal ease, as is evidenced by Zernoff's (1931) failure to initiate their production in *Carausius morosus* Brunn.

Although not an antibody, it might be mentioned here that *complement* (a thermolabile component of serum necessary in lytic reactions in vertebrates) has not as yet been demonstrated in insects.

Bactericidal substances (*bactericidins*) act upon bacteria. They usually kill the bacteria or at least inhibit their growth, but they do not lyse or dissolve them. One of the first demonstrations of this killing power of insect blood was Glaser's (1918) observation that, 10 days after inoculation, the immune hemolymph of the grasshopper *Melanoplus femur-rubrum* (DeG.) was capable, in vitro, of killing "*Bacillus poncei*" Glaser. This bactericidal property of immune hemolymph apparently cannot be attributed to substances formed in the hemolymph itself or produced by the hemocytes in the course of an infection.

The blood of the large milkweed bug, *Oncopeltus fasciatus* (Dall.), contains an antibacterial agent active in vitro against *Micrococcus pyogenes* var. *aureus* (Ros.) Zopf [*Staphylococcus aureus* Ros.] and at least one strain of *Bacillus subtilis* Cohn *emend.* Praz. According to Frings, Goldberg, and Arentzen (1948), the active principle is water-soluble, is stable to boiling for 30 minutes but is destroyed by autoclaving and by prolonged standing at room temperature, passes through a bacteriological filter, and is active at a dilution of at least 1 part in 10,000. The exact nature of this antibacterial property of *Oncopeltus* blood has not been determined. Nor is it known if it has any relation to the type of activity demonstrated by Glaser with grasshopper blood against "*Bacillus poncei*" or by Olivier (1947) with an acetone extract of macerated wax-moth larvae against tubercle bacilli.

Agglutinins are antibodies that act upon foreign cells in such a manner as to cause them to gather together in aggregates or clumps (*i.e.*, agglutinate) and to settle out of suspension. The presence of agglutinins in insect blood has been questioned by some workers, but Glaser (1918) emphatically claims to have demonstrated them in the blood of the same species of grasshopper in which he found bactericidins. The blood was tested 2 weeks after the bacteria had been inoculated, and in hanging-drop preparations the organisms agglutinated in 20 to 30 minutes. Similar

results have been reported by Gary, Nelson, and Munro (1948), who obtained agglutination reactions against *Bacillus larvae* White in the hemolymph of honeybees taken from colonies suffering with American foulbrood. As determined by a series of agglutination reactions, an increase in resistance from young larvae to the adult bee was observed. Agglutination of *Bacillus subtilis* Cohn *emend.* Praz. was also seen, but this is believed to be nonspecific.

Precipitins are antibodies that react with foreign proteins in solution, aggregating the molecules with the formation of a precipitate. A few cursory tests for precipitins in the blood of immune insects have been made, but mostly with negative results. However, if agglutinins and other antibody manifestations are present, it would seem entirely likely that precipitins are present also.

Antitoxins act upon those poisons, such as exotoxins, which are protein in nature. Their primary action is to neutralize the toxic or poisonous qualities of these toxins. Although very little work has been done to demonstrate the presence or formation of antitoxins in insects, there are good indications that such exist. Chorine (1929, 1931), for example, has found diphtheria toxin to be toxic for caterpillars of the wax moth, *Galleria mellonella* (Linn.). He was also able to bring about an immunity of the insects to the toxin through the administration of a toxoid, *i.e.*, a detoxified toxin.

Opsonins are antibodies that act upon foreign cells, sensitizing them in such a way as to cause them to be readily engulfed by the phagocytes. As was stated earlier in this chapter, opsonins have not yet been demonstrated in insect blood, but it is not unlikely that they occur.

Neutralizing or protective antibodies are antibodies that neutralize the infectiousness of an infectious agent, usually a virus. To demonstrate this, the immune serum is mixed and incubated with the virus, after which the mixture is inoculated into a susceptible animal. If neutralizing antibodies are present, the virus is rendered noninfective. As yet, neutralizing antibodies have not been found with certainty in the blood of insects. It has been shown that certain numbers of some species of caterpillars show an "immunity" to virulent polyhedral viruses; whether this represents insusceptibility or true neutralization of the virus has not been elucidated.

Hypersensitivity is the manifestation of an antigen-antibody reaction within the body of an animal which shows a heightened reaction to a subsequent introduction of substances which, when first introduced, provoked little or no reaction. Hay-fever allergy or the appearance of hives after eating strawberries are classic examples in man. The term "anaphylaxis" is used to designate severe forms of hypersensitiveness in experimental

animals, and usually under unnatural circumstances. Indication that these phenomena may take place in insects is provided by the observations of Metalnikov and Gaschen (1921*b*). They found that larvae of the wax moth, *Galleria mellonella* (Linn.), after the administration of a vaccine of cholera vibrio, are immune to the minimum fatal dose, but they succumb more rapidly to larger doses than do untreated larvae. This reaction appears, on the surface at least, to be of the nature of hypersensitivity or an anaphylactic shock.

Actively Acquired Immunity

It has been fairly well demonstrated that, when stimulated by the proper antigens, insects are fully capable of actively producing the corresponding antibodies. This active immunity may of course be brought about naturally when the insect acquires the infection or disease through natural agencies, or it may be initiated artificially.

Naturally Acquired Active Immunity. It is somewhat surprising that so few observations have been made on the presence of naturally acquired active immunity in insects. This is true from the standpoint of immunity in both individual insects and in insect populations. We know practically nothing, for example, concerning the residual immunity in insects that have survived an epizootic wave. Do acquired immunities build up and diminish in nature as the result of exposure to infectious agents? What percentage of cases of apparent natural or normal immunity is actually the result of earlier exposure to the infectious agent concerned? All we can do at present is to speculate on the answers to these and other similar questions.

One of the few instances in which naturally acquired active immunity has been found present in insects in nature was that demonstrated by d'Herelle in 1911. He showed that 20 or 25 per cent of the grasshoppers in epizootic areas had acquired an immunity to "*Coccobacillus acridiorum*" d'Her.

Artificially Acquired Active Immunity. The principal methods by which immunity may be artificially induced include the inoculation of insects with (1) minute doses of virulent organisms, (2) old attenuated cultures, and (3) cultures heated to 60°C. The production of an acquired immunity by these methods is not always successful but varies with the insect and with the microorganism concerned. For some unexplained reason certain insects apparently cannot be immunized at all by ordinary methods. Others respond well, although in most cases this acquired immunity in insects is not very strong or complete. Most immunized insects can survive several lethal doses of the virulent organisms, but no extra heavy doses. Some workers have observed that when extra heavy

doses are given immunized insects they frequently die sooner than do unimmunized individuals given the same dosage. On the other hand, when immunity is produced it generally arises in an extremely short period of time—usually within 24 hours following a single injection of an old culture or vaccine.

The literature contains mention of numerous instances in which active immunity has been produced in insects using a variety of microorganisms, mostly bacteria. The insects most frequently used in these attempts include the European corn borer, cutworms, and larvae of the wax moth. The bacteria include such species as *Escherichia coli* (Mig.), *Proteus vulgaris* Hauser, *Bacterium melolonthae non-liquefaciens* Pail., and several bacteria, such as *Vibrio comma* (Schroeter) and *Salmonella enteritidis* var. *Danysz* Bahr, foreign to insects. To be sure, it is unlikely that the lepidopterous insects used in most of these experiments would ever naturally suffer from infections by organisms (such as the last two just cited) ordinarily causing disease in man. Nevertheless, by using such bacteria, of low virulence for insects, early investigators, particularly Metalnikov and his associates, were able to demonstrate that bacterial antigens were capable of eliciting an immunity response in insects. Paillot showed the same phenomena to exist when the more virulent insect pathogens were used as attenuated cultures. Not all bacteria, however, have served as efficient antigens in insects, and acquired immunities to protozoa and fungi are also difficult to produce. Some of the idiosyncracies of immunization no doubt lie with the insect too, as is evidenced by the fact that the ordinary colon bacillus, *Escherichia coli* (Mig.), serves as an effective antigen in the caterpillar of the wax moth, *Galleria mellonella* (Linn.), while all attempts to immunize the oriental cockroach, *Blatta orientalis* Linn., with this same organism have failed.

Considerable use has been made of vaccines as immunizing agents. Both living and killed vaccines have been successful in this regard (Paillot, 1920; Metalnikov and Gaschen, 1921*a*; Ishimori, 1924; Glaser, 1925; and Metalnikov and Chorine, 1929). The vaccines may be heat-killed or chemically killed, and the living vaccines may consist of cultures attenuated by age, dissociation, or other methods. In general it has been found that small doses of vaccine bring about an immunity more quickly than do large ones. In many insects the immunity produced during the larval stage persists on into the adult stage. As is usually the case with higher animals, vaccines administered to insects via the oral route fail to immunize.

The degree of specificity exhibited in most cases of acquired immunity in insects does not appear to be very great. Although a stronger and more stable immunity against a particular microorganism usually results from

the use of a specific vaccine, there frequently is a marked immunity against heterologous organisms. Ishimori and Metalnikov (1924), for example, were able to immunize *Galleria mellonella* (Linn.) against *Vibrio comma* (Schroeter) by inoculating them with *Bacillus anthracis* Cohn and *Escherichia coli* (Mig.), and with Chinese ink. A more efficient immunity, however, resulted from the use of the specific vaccine. Contrariwise, *Micrococcus galleriae* No. 3 I. & M. and *Bacterium galleriae* Chor. elicited a stronger immunity against *Vibrio comma* than this organism did against itself. Zernoff (1934) and other investigators have obtained similar results.

Mechanisms of Acquired Immunity. That school of thought dominated by Metalnikov insisted that the mechanisms responsible for acquired immunity in insects were concerned with the activity and sensitivity of the phagocytes. Upon the introduction of a bacterial vaccine, these blood cells adapted themselves to the new conditions and presumably became more aggressive and active. In other words, the increased immunity following the introduction of the proper antigens was actually the result of an increase in phagocytic activity. To a certain extent, this has been found to be true in most animals. That it is the whole story, however, was soon challenged by other investigators, particularly Paillot.

Paillot was firmly convinced that the humoral reactions are of much greater importance in insect immunity than are the cellular reactions, which play a secondary role. He believed that most of the protective power of the blood is the result of its bactericidal and bacteriolytic properties. In the immune insect the invading bacteria are disintegrated into small granules. When this process is well under way, the intensity of phagocytosis increases and the granules are engulfed and destroyed.

On the basis of the meager information available, the view that both humoral and cellular factors are important in the immunity processes of an insect would seem to be the most logical. At least it appears to be a safer conclusion than the assumption that either one alone is the exclusive mechanism.

Just which tissues, systems, or substances of the insect's body determine the nature or intensity of the humoral and cellular immunity has not been thoroughly established. Nor do we know whether the site of antibody production is limited to one or several tissues or organs of the body. The elucidation of this point in insects would probably have important applications to the same problem in the case of vertebrate immunity. Interesting attempts to determine some of these basic mechanisms have been made, using insect larvae. Metalnikov (1927), for example, found that, when the cerebral ganglia and the first and second thoracic ganglia of *Galleria mellonella* (Linn.) larvae were destroyed with a hot needle, the caterpillars could be immunized against the cholera vibrio and the bacillus

of Danysz as easily as if they were normal insects. Similar results were obtained when the larvae were decapitated at the second thoracic segment. If, however, the third thoracic ganglion were destroyed, a serious operation for insects, the animals could not be immunized, and there occurred a decrease in the natural resistance of the caterpillars to staphylococci. There was also a decrease in the number of leucocytes, and in the phagocytic index, which, according to Metalnikov, permitted the test bacteria to multiply rapidly and to kill the host.

If ligatures are placed tightly around the mid region of wax-moth caterpillars—a condition they can withstand 2 or 3 weeks—it is possible to immunize the two portions of the insect separately. When the anterior half is immunized, this immunity is transmitted to the posterior half. On the other hand, immunization of the posterior half does not afford protection to the anterior half. Metalnikov explained this paradox on the grounds that in the caterpillar the anterior half of the central nervous system is concerned with immunization and that the immunity is transmitted through the ventral chain of ganglia. He believes that this hypothesis is supported by his observation that, if the ventral chain is first destroyed by the application of a hot needle at the mid region, immunization of the anterior half of the ligatured insect no longer confers immunity on the posterior half. Before accepting Metalnikov's conclusions it appears that further experimentation is required to ascertain the possible role played by the blood supplying the living nerve tract.

Metalnikov has also maintained that acquired immunity may be hereditarily transferred from one generation of wax-moth caterpillars to the next. His experimental results may more logically, perhaps, be interpreted to represent a case of the natural selection of naturally resistant individuals which increased from one generation to the next. The more susceptible individuals would die off and fail to pass their higher degree of natural susceptibility on to the next generation (Huff, 1940).

The exact mechanism by which certain insects acquire a resistance to chemical insecticides is not known. Nevertheless it is an established fact that scale insects, for example, develop races that are markedly resistant to the effects of certain sprays and fumigants. Natural selection appears to be an important factor in the development of these resistant races.

Passive Immunity

As in vertebrate animals, an immunity acquired in one insect may be transferred passively to another. This is usually accomplished by the transference of blood or serum from the immunized animal to the circulatory system of the animal to be immunized.

Passive immunization in insects has been attempted in only a few instances. Typical of the results that might be expected with most insects are those obtained by Zernoff (1928*a,b*) with larvae of *Galleria mellonella* (Linn.). He found that the blood of caterpillars actively immunized against the Danysz bacillus (*Salmonella enteritidis* var. *Danysz* Bahr) could be transferred to normal caterpillars, immunizing the latter not only against the Danysz bacillus but to even a greater extent against "*Coccobacillus acridiorum*" d'Her. On the basis of this observation Zernoff concluded that passive immunity is not accompanied by any marked degree of specificity. Usually, however, the passively conferred immunity is more intense with the specific organism than with others, although a considerable degree of nonspecificity remains. Passively acquired immunity in insects is also characterized by its relatively short duration, although a temperature of 80°C. for 30 minutes does not entirely destroy the immunizing property of the blood. According to Zernoff, either the hemocytes or the hemolymph (plasma) used separately will confer passive immunity, as well as does the whole blood itself.

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CHAPTER 8

SYMPTOMS AND PATHOLOGIES

This chapter will be largely one of descriptive terms and definitions. Although the student probably will not find it thrilling reading, he should find in it an orientation to the numerous types of change that occur in insects. The terminology that has developed during man's study of disease processes is such that it requires a certain amount of defining if we hope to understand the descriptive terms used in writing about diseases and their pathologies.

Like other animals, insects afflicted with diseases or injuries usually exhibit behaviors and changes more or less characteristic of the disorder from which they are suffering. In general, the abnormal variations in behavior are spoken of as "symptoms," and the changes in bodily structure brought on by disease are known as "pathologies." To be sure, there is some overlapping in this terminology, as is evidenced by the fact that certain obvious pathologies are sometimes considered as symptoms. A particular combination, set, or sequence of symptoms is called a "syndrome" and usually characterizes a particular disease or infection.

CLASSIFICATION OF SYMPTOMS

The symptomatology of insect diseases is not always easy to ascertain. Indeed it is often difficult, in insects, to distinguish the normal from the abnormal. Changes in the behavior and bodily appearance of infected insects may vary under different environmental conditions even though the inciting cause of the disease remains the same. The chief difficulty is the fact that, in general, the symptoms occurring in infected insects have not been at all well studied or catalogued. Any classification of symptoms that we may present here is of necessity purely tentative and far from complete. The following paragraphs, however, perhaps may assist the student in properly orienting his observations.

Movement and Irritability. As in the case of most diseased animals, diseased insects usually become generally lethargic and sluggish in movement after the disease is well developed. They usually respond less rapidly and less actively to external irritants and when moribund may be devoid of practically all movement. Sluggishness is not always the case, however, and sometimes, especially in the very early stages of some diseases, the

insects may be markedly restless and irritable, some buzzing about noisily but aimlessly, obviously exhibiting increased irritability. Infected insects often show marked muscular contortions and contractions, frequently characterizing the type of infection involved.

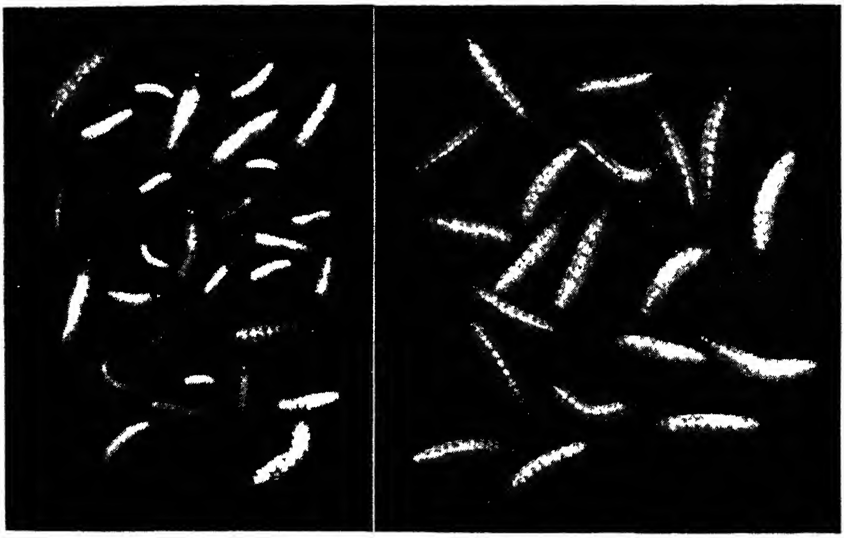
Diseased insects sometimes show characteristic "migrations" or movements from one position to another. Thus, in the case of certain virus infections (*e.g.*, *Wipfelkrankheit*), the infected caterpillars climb to the tops of the trees, or other host plants, where they hang by their prolegs and die. Exactly what it is that prompts movements of this kind is not known, although one or two theories (insects seeking more suitable humidity and temperature conditions, for instance) have been advanced. From the standpoint of the disease, this movement facilitates dissemination of the infectious agent, which may fall or be washed down from the disintegrating insect onto or over the foliage, there to be contacted by healthy individuals.

Discoloration. With many diseases, the affected insect assumes a characteristic coloration that distinguishes it from a healthy individual. This change in color may occur uniformly over the specimen, or it may occur only in spots or blotches. It may be the result of changes within the tissues themselves, or it may be due to the mere presence of the infecting organism. Thus a dark brown to black coloration may result from the breakdown of tissues subjected to the enzymatic action of bacteria. Or the bacteria themselves may possess a pigment that is imparted to the insect after the bacteria have increased to sufficient numbers for their presence to be grossly detectable. This sort of coloration is frequently seen in insects infected with red-pigmented *Serratia marcescens* Bizio, which imparts a bright red color to its host. Sometimes the presence and accumulation of bacterial or protozoan spores may give the insect a white opaque appearance, as seen in the milky diseases of grubs and the microsporidian diseases of mosquito larvae.

Some types of color change, such as the jaundiced or yellowish appearance of certain lepidopterous larvae infected with polyhedral viruses, are only imperfectly understood. In fact, often it is not altogether clear just what is responsible for the brown to black coloration of the integument or underlying tissues of insects infected and dying with many of the bacterial and virus diseases. Some authorities have assumed that this discoloration is due to the liberation of oxydases. The fact that diseased larvae usually turn black very quickly after death, or show black spots while still living, is thought to be caused by the increased production of oxydizing enzymes accelerated by the insufficiency of nutrition. The only well-known distinctly black pigment not derived from hemoglobin that is produced in living tissue as the result of specific cell activity is an organic substance known as "melanin," which, in higher animals at

least, is the cause of pigmentation in certain types of tumors and other pathological conditions.[1] (When black, melanin is properly called "fuscin.")

The brown to black spotted areas seen on larvae suffering from certain microsporidian diseases, such as pebrine, have been explained by the coagulation of blood after a hemorrhage takes place through wounds



A B

Fig. 66. General appearance of larvae, *Gnorimoschema operculella* (Zell.), suffering from protozoan (microsporidian) infection. Note the variation in size and color of infected larvae in *A* as compared with these characteristics of normal larvae as shown in *B*. (*Photographs by K. M. Hughes.*)

caused by the parasites in the integument, and by the belief that the microsporidian spores enclosed in the area turn yellow in color because of the lack of oxygen. It also appears that the cuticula itself turns brownish in color over the infected hypodermal cells.

Changes in Size and Shape. A not infrequent indication of disease in insects is the size and shape of the affected animals, as compared with healthy individuals. Whereas most of the larvae of a single healthy brood, held together under identical conditions, grow and develop at about the

[1] The pigment melanin is insoluble in water, alcohol, ether, chloroform, carbon bisulfide, and weak acids, but it is dissolved in alkalies, strong acids, or boiling alcohol. Accordingly, sections of diseased larval tissue should sometimes be passed through an alcoholic solution of potassium hydroxide before staining in order to remove the pigment should it be melanotic in character. In some cases, the caustic potash then dissolves the pigment in the infected areas, allowing the microorganisms and surrounding tissues to take stains.

same rate, those of a diseased brood frequently develop at varying rates, so that some of the individuals remain small while others grow at a normal rate. In addition to being stunted in growth, infected larvae are often shriveled in appearance, and the normal turgidity and plumpness may be lost. The entire body may take on an inflated or swollen appearance, or parts of it may be distended with microorganisms or products resulting from disease. Tumorlike protrusions and other deformations may also occur. The various appendages, especially the legs and wings, may have appearances slightly modified from that of the normal, or they may be distorted or held askew.

Digestive Disturbances. With some types of disease the occurrence of certain digestive disturbances is a characteristic symptom. Most seriously infected insects have a reduced appetite and often refuse to eat or to be attracted by food. As the disease progresses the insect may vomit or regurgitate its food, or there may be simply an oozing of watery fluid from its mouth. If the infection is one centered mainly in the digestive tract, there is very likely to be evidence of diarrhea, with fluid sticky discharges from the anus. On the other hand, some infections are accompanied by a hardening of the intestinal contents, *i.e.*, constipation. This is sometimes evidenced by the rate at which the feces are discharged—usually slowly and apparently laboriously.

Other Abnormal Physiological Reactions. Inasmuch as the process of disease is essentially one characterized by an abnormal physiology or metabolism on the part of the host, it is not strange that, in addition to digestive disturbances, other abnormal physiological reactions should manifest themselves as part of the symptomatology of many diseases. Indeed these physiological changes are many and varied, and those mentioned here are probably only those which are the most obvious.

The inability of diseased larvae to pupate or of pupae to become adults or of adults to lay fertile eggs are rather common occurrences among diseased insects. The same may be said with regard to the inability of infected insects to carry on the normal functions of excretion, secretion (such as the spinning of silk), and the like. Sometimes the destruction of certain nerves or ganglia causes a malfunctioning of a certain part or parts of the body. Disease may also cause an abnormal sensitivity or lack of sensitivity to such things as heat, light, touch, and other physical agencies.

The symptomatology of some diseases may include changes in the consistency or appearance of certain body tissues. Thus tissues may liquefy or become thickened, or the chitinous cuticula may become excessively brittle. The body fluids may become cloudy, turbid, or milky—usually because of the presence of microorganisms. Chemical and cellular changes in the blood may also occur, but these have not been well studied.

Although many dead insects give off odors of putrefaction, insects with certain diseases have characteristic odors (*e.g.*, the "gluepot" odor of American foulbrood). The instances in which the odor may serve as an indication of a specific infection, however, are few.

Post-mortem Changes. Correctly speaking, the post-mortem changes, *i.e.*, the changes that take place after death, are not a part of the symptomatology of the disease in an individual animal. In insect diseases, however, the post-mortem changes apparent in that part of the population which has died off are frequently a revealing indication or diagnostic sign as to the nature of the disease still raging in the living hosts. For example, the fact that insects which have succumbed to polyhedral-virus diseases frequently disintegrate into a liquefied mass is a characteristic of great diagnostic importance. The mummified appearance of insects dead of fungous diseases is of similar significance.

Post-mortem changes usually take place very rapidly after the death of the insect. The tissues are rapidly destroyed by autolytic enzymes and by the rapid growth of adventitious bacteria. Upon the death of the insect, the bacteria normally in the gut frequently bring about the rapid destruction of all internal tissues. These bacteria may, in fact, overgrow the pathogenic microorganisms that brought about the insect's death, thus making difficult an accurate diagnosis.

Low refrigerator temperatures may to some extent retard the post-mortem changes, but examination of dead insects should in most cases be made as soon as possible after death has taken place. This is especially true of histological examinations, which are preferably made during the earlier stages of the disease or just before death. Certain bacteriological examinations and those for fungi, protozoan spores, and polyhedral bodies may frequently be accomplished after the host has been dead for some time —several hours or days. Indeed many entomogenous fungi do not fully develop or produce spores until after the death of the host insect. After a dead insect has become dried there is usually very little that can be done with it in the way of making a diagnosis—except in the case of fungi and other persistent microorganisms. Even these examinations must be made very carefully to avoid mistaking the true cause of the insect's death.

In determining whether or not certain changes are true pathological changes or merely nonspecific post-mortem changes, one must be careful in what one recognizes as a criterion of death. Some workers consider an insect to be dead when it no longer reacts to external stimuli. Others maintain that an insect is dead only when it no longer yields blood, as for microscopic smears. In an earlier chapter it was pointed out that, like other animals, an insect does not die all at once. Some tissues die before

others, and some tissues may be dead and undergoing autolytic changes before all external signs of life are gone. This fact alone makes the work of the histopathologist particularly precarious when it is necessary to describe certain of the finer alterations in diseased tissue. It is not always a simple matter to differentiate true pathological changes from post-mortem changes.

PATHOLOGICAL PROCESSES

The pathological changes found in diseased insects may generally be described as either of two types: gross pathologies and histopathologies. *Gross pathologies* include those abnormal changes which occur in injured or diseased organs and tissues, and which can be detected and observed without the aid of a microscope. They usually involve such abnormal changes as those in size, shape, color, and appearance, as well as those in the consistency of tissues and organs. *Histopathologies* deal with pathological changes in tissues and cells as seen microscopically. They are usually more significant than are the gross changes; but, unfortunately, there is a great gap in our knowledge of this subject as it pertains to the diseases of insects, leaving it to be one of the principal branches of insect pathology most in need of study.

Pathological processes manifest themselves in a variety of ways, and one must usually seek their explanation by means of cellular and histological examinations. By such methods one is able to detect most retrogressive changes that occur during the course of a disease. These are simply changes in which the affected tissue has gone backward from normal and is temporarily or permanently less capable of doing its work. Examples are the two processes infiltration and degeneration.

Infiltration. The term "infiltration" is used in referring to the deposition within or between cells or tissues of an abnormal substance or of an excess of a substance that is normally present. In mammals the substances commonly deposited are fat (oil), amyloid (lardacein, a starchlike substance), pigment, glycogen, and lime salts. These are spoken of as fatty, amyloid, pigmentary, glycogenic, and calcareous infiltration. Sometimes certain abnormal or malignant cells may insinuate themselves among normal cells; this too is a form of infiltration.

To what extent these types of infiltration occur in insects is not well known. It appears likely that more instances of this kind of retrogressive change will be found in insects as the histopathology of these animals becomes better known. Incidentally, it should be remembered that insects characteristically deposit certain excess materials in certain cells of the body for storage or for ultimate elimination (*e.g.*, oil droplets in the fat cells, uratic concretions in adipose and other tissue cells, etc.).

Degeneration. Degeneration is a retrogressive change in living tissue,

and is to be clearly differentiated from necrosis, which refers to the death of cells. The living, functioning substance of the tissues becomes *replaced* by a new material that is inert, the result being that the tissue ceases to perform its duties in proportion to the extent to which the change has taken place; *i.e.*, during the time this condition lasts, the affected cells are below normal in function and structure. A cell may degenerate and later recover, returning again to normal. If, however, the cause persists, the degenerative process will persist and usually increase in degree. If the increase continues, the cells become so disturbed in structure and function that they die.

Several types of degeneration are recognized in mammalian tissue, but which of these and to what extent they occur in insects remains to be determined. *Parenchymatous* or *granular degeneration* occurs particularly in epithelial cells. It may be caused by poisons of all kinds, including those produced by microorganisms and other parasites. The affected cells swell, and their protoplasm becomes granular. The granules, which some believe to be particles of protoplasm that have undergone coagulation or condensation, are somewhat refractile and do not take ordinary stains. The effect is one that lessens the function of the cells—whatever this function happens to be. *Fatty degeneration* is characterized by an accumulation of fat or oil in the epithelial cells and possibly in the fat cells. The causes are essentially the same as those cited for granular degeneration, which may precede most cases of fatty degeneration. *Colloid degeneration* refers to a condition in which the cells contain a ropy, gelatinous or sticky substance that has no structure and is colorless or yellow to brownish in color. *Mucoid degeneration* occurs in either epithelial or connective tissues and is characterized by the formation of a substance containing mucin, a glycoprotein. The term *hyaline degeneration* is applied to a condition in which the tissues lose their normal structure and assume a uniform glassy appearance. *Hydropic degeneration,* or cellular edema, is actually an edema of the cells and occurs in inflammatory processes. The cells become swollen and contain vacuoles filled with fluid. Epithelial cells are chiefly affected.

Necrosis. The local death of cells or tissues, as distinguished from the death of the entire insect body, is called "necrosis." To the naked eye, necrotic tissue is usually gray or yellow in color, but it may be darkened by blood content, pigment, or oxidation to any shade, even black. Microscopically, necrosis has two characteristic features: (1) the outlines of individual cells and other tissue constituents are lost or almost obliterated, and the tissue appears homogeneous or uniformly granular; (2) differential staining is lost, all kinds of tissue and all parts of cells staining the same color by any individual stain. As a rule, necrotic tissue stains poorly

by most methods, although acid dyes, such as eosin which stains the tissue brilliant pink, usually give the best results. When necrotic tissue is examined microscopically, the absence of nuclei is one of the first things to be noticed.

Immediately after the death of the tissue or cell, there may be no apparent change. A short time thereafter, however, autolysis sets in, and the intracellular proteases begin to split up the proteins of the cell. If these ferments are destroyed (as in tissues killed by heat or acid) the lysis of the dead cell proceeds at a slower rate, since the proteolytic enzymes must then be furnished by the hemocytes or other cells. The chromatin of the nucleus also undergoes disintegration, apparently with the liberation of an increased amount of nucleic acid, since, for a while, the nucleus stains more intensely with basic dyes (*pycnosis*). As the chromatin decreases in amount the nucleus fades (*karyolysis*) until finally it does not take the basic stains at all, although its form may still be retained. Sometimes the nucleus breaks apart in fragments (*karyorrhexis*), although this does not happen nearly so often as does karyolysis. Cytoplasmic changes are also recognizable. The cytoplasm may take on a solid homogeneous appearance with the loss of its characteristic granular or reticular structure, or varied types of fragmentation and liquefaction may occur. (In the study of material from dead insects, care is necessary in distinguishing these changes from those due to post-mortem processes.) Unless it occurs immediately as the result of agents or poisons that in themselves cause death, necrosis is almost always preceded by degeneration which proceeds until the disintegrative changes take place in the cytoplasm, nuclei, and membranes of the cells. This slow death, in which the cells pass through degenerative changes, is sometimes referred to by the term *necrobiosis*.

All these changes in necrotic cells are caused by chemical processes occurring in them during and after the time they die. That some of these chemical processes are of the nature of changes brought about by intracellular enzymes is indicated by the fact that necrosis occurs in aseptic as well as in infected areas.

In vertebrate pathology the various types of necroses have been given names, thus: coagulation necrosis, liquefaction necrosis, caseation necrosis, and fat necrosis. It is possible that the latter two types do not occur in insects, except in rare instances. On the other hand, coagulation necrosis (necrosis accompanied by the coagulation of fluid within the affected tissue) and liquefaction necrosis (in which the necrotic tissue liquefies) are probably more common.

The term "gangrene" is applied to necrotic tissue that undergoes putrefaction while it is still attached to the body. In some bacterial

diseases of insects the limbs become very necrotic and may be said to be gangrenous. Since gangrene is usually caused by a diminution or stoppage of circulation, the term is perhaps not always strictly applicable to conditions in insects. Nevertheless such a circulatory disturbance can occur in parts of the insect's body, as is evidenced by that which occurs in Japanese beetle type B milky disease and certain other bacterial diseases of grubs. The term may also be applied to such a disease as that of the Lychee stink bug, *Tessaratoma papillosa* Drur., in which the legs and antennae darken and drop off, apparently as a result of the action of a fungus.

Other Pathological Changes. Our meager knowledge of the pathological changes that occur in insects makes it impractical to review here all the changes that might possibly present themselves to an observing insect pathologist. It does seem pertinent, however, to consider briefly a few of the most probable manifestations of disease as they may be observed in insect tissues.

One gross pathological change that is seen from time to time in insects is that designated by the term *hypertrophy*. From the histopathological standpoint it is seen frequently in diseases caused by all major groups of microorganisms. This change is characterized by an increase in size (weight) and functional capacity of an organ, tissue, or cell. A hypertrophied organ or tissue does more work than does a normal one. In fact, the cause of hypertrophy is a gradually increasing demand for more work; it does not develop suddenly but requires time. It should not be confused with *hyperplasia*, a progressive tissue change consisting of increased growth or formation, but not an increase in function. Hypertrophy is essentially the opposite of *atrophy* in which the affected cells undergo degenerative and autolytic changes, become smaller, and have a lessened functional capacity. The atrophied tissue or organ is generally diminished in size. The cytoplasm of the individual cells shrinks, but usually without any conspicuous degenerative changes. Atrophy occurs under various conditions of altered physiology, disuse, starvation, and the like. An organ or tissue that is not made small by atrophy but never reaches normal size is an example of *hypoplasia*. It is undergrowth and a type of malformation. *Aplasia* (or agenesis) refers to the entire failure of organs to develop. Such organs or tissues are usually designated as just being absent. *Metaplasia* means a change of growth or formation and is applied to a change of tissue from one form to another but within the same type. For example, epithelial cells may change from one form to another (squamous cells to columnar cells) but never to connective tissue cells. The term *heteroplasia* signifies dissimilar growth and applies to cases in which a particular type of tissue not normally found in an organ occurs there.

Circulatory disturbances are not known to be of great consequence in most insect diseases. Such things as *hemorrhage* (the escape of all the constituents of the blood) of course occur when a leg or wing is pulled off or the body wall is otherwise broken open. It may occur from injury, from leakage without definite openings, by erosion, and by the opening of the body wall and tissues during "ulcerating" or necrotic processes. Although probably not of great significance in most insect diseases, occasionally internal "clots" of blood cells and hemolymph occur. What might be called a "thrombus" is formed, and a pathology known as *thrombosis* results. Possibly other emboli (*i.e.*, bodies floating in the hemolymph), such as clumps of microorganisms, may also cause trouble by plugging narrow passageways, sinuses, or appendages. An excess of hemolymph (without the hemocytes) in cells or in any of the small spaces of the insect body may be spoken of as an *edema*. If the hemocytes also are present, the words *hyperemia* or *congestion* are probably more explicit. A general deficiency of blood is termed *hypoemia* or *oligemia*. The term *anemia* (not used here in the physiological sense of a deficiency of hemoglobin) is used in pathology to indicate a circulatory change in which there is a lessened supply of blood in a local area. The blood or hemolymph itself may be perfectly normal, but the proper amount does not get into the affected part. To emphasize the difference, this local anemia is sometimes called *ischemia*. If the anemia is of short duration (minutes), no recognizable damage results; if it persists, the tissues will undergo atrophic or degenerative changes, or both, and later necrosis. The word *stasis* indicates the complete stoppage of blood in a part or in the entire insect.

Inflammation in insects does not manifest itself in exactly the same way as it does in higher animals, and, in fact, the same phenomenon, as we know it in vertebrates, probably cannot be said actually to occur in Hexapoda. In the broad sense, however, and in the pathological changes it brings forward, insects do undergo what might be thought of as an inflammatory process. Inflammation is simply the local reaction of tissue to injury or to the action of an injurious agent. In most cases it is a beneficial and highly desirable process and does not occur when the injury has overwhelmed the tissues. Phagocytosis, as well as humoral immunity principles, may be important parts of the process. Inflammation is usually terminated by one of three processes: (1) resolution, a return of the tissues to normal in which there is no tissue loss; (2) regeneration, a return to normal in which there has been a loss of tissue but this loss has been replaced; (3) repair, which occurs when there has been considerable tissue damage; the destroyed tissue does not regenerate, but it does heal.

CHAPTER 9

BACTERIAL INFECTIONS

From the standpoint of total numbers, bacteria constitute the most abundant type of microbiota associated with insects. It is not surprising, therefore, that a significant number of these microorganisms have been found causing infections in insects under a wide variety of conditions.

The bacteria pathogenic for insects are, in general, no different from most bacteria in their basic characteristics. Since the majority of students using this book have probably had courses in bacteriology or will at least be familar with the general nature of bacteria, our remarks in this regard will be limited. Suffice it to say that bacteria may, in general, be considered as one-celled plantlike organisms that multiply by fission. This, of course, is a broad concept to which there are exceptions. They may be distinguished from most other microorganisms by their much smaller size and by the fact that the existence of a distinctly formed nucleus is not readily discernible, although an increasing amount of evidence indicates that probably all bacteria have a chromatin body analogous to a nucleus.

Bacteria belong to the class Schizomycetes, which is usually divided into five or more orders, depending upon the system of classification followed.[1] From the standpoint of insect pathogens, nearly all the bacteria with which we shall be concerned in this chapter belong to the order Eubacteriales, suborder Eubacteriineae. This group consists of simple undifferentiated cells, although many of the species are pleomorphic. They may be motile or nonmotile, and endospores are present in one family (Bacillaceae). They are frequently chromogenic. Most of the bacteriá pathogenic for insects are confined to the following six families: Bacillaceae, Enterobacteriaceae, Bacteriaceae, Lactobacteriaceae, Micrococcaceae, and Pseudomonadaceae. Our discussion will be divided according to these families and in the same order.

General Characteristics of Bacterial Diseases. In general, insects suffering with bacterial disease exhibit a lack of mobility, a diminished appetite, and rectal and oral discharges. In most cases the infecting bacterium eventually invades the body cavity of the insect, and infection

[1] In the present volume, the systematics of the sixth edition of "Bergey's Manual of Determinative Bacteriology" are followed, since most American workers are familiar with its system of classification.

ends in a septicemia. After death, the body (of a larva especially) darkens rapidly to a brown or black color. It is usually very soft and becomes more or less shapeless. Thé internal tissues may break down to a viscid consistency, sometimes accompanied by odor, but ordinarily they do not "melt" or liquefy to the extent characteristic of certain virus infections. Usually the insect dries and becomes shriveled, with the integument remaining intact. Smears or histological sections of an insect dead or dying of a bacterial disease usually show large numbers of the causative bacterium present.

One unfortunate situation with regard to the diseases of insects has to do with the nomenclature used in referring to them. Names based largely on the symptoms exhibited are likely to be confusing. For example, several bacteria are capable of producing a septicemia in cutworms; hence the name "cutworm septicemia" would not be specific or clear. Yet, in the absence of a more satisfactory terminology, such nomenclature is convenient and continues to be used by many authors. In distinguishing between different diseases, the important thing to keep in mind is the specific etiologic agent involved, and the use of generalized names without reference to the specific causative agent should be discouraged.

BACILLACEAE INFECTIONS

The family Bacillaceae consists of two genera: *Bacillus* and *Clostridium*. All species of these genera form spores. Members of the genus *Bacillus* are aerobic, while those of the genus *Clostridium* are anaerobic. Of the two genera, only *Bacillus* contains species that have been found infecting insects in nature. Experimentally, *Clostridium novyi* (Mig.) and *Clostridium perfringens* (Veil. & Tub.) have been found to be somewhat pathogenic for the wax moth, *Galleria mellonella* (Linn.). No clostridia, however, have as yet been reported as natural pathogens for insects.

Nomenclature. In the past much of the literature in insect pathology has been burdened with a confused taxonomic and nomenclatorial situation as concerns those members of the genus *Bacillus* which cause infections in insects. There have been several reasons for this state of affairs, one of them being the frequent lack of understanding of basic rules of nomenclature and systematic arrangement. Another reason has been the absence, until recent years, of a satisfactory system of bacterial systematics to guide even the specialists in this genus. As a result much of the literature is filled with bacterial names that are entirely inappropriate and incorrect. In 1946 an attempt was made (Steinhaus, 1946*a*) to segregate from the genus *Bacillus* those species which do not belong in this category by virtue of their nonsporogenic character. For a list of those species which have

been given the generic name *Bacillus* but which, because they are not sporogenic, are incorrectly placed in this genus, and for a list of bacterial pathogens of insects that have been characterized as forming spores, the reader is referred to the publication just mentioned. Further consideration of the information on which these lists were based makes it possible to reduce the number of names of those entomogenous species which may find acceptance as valid and distinct species of the family Bacillaceae to a mere handful, which would include the following:

Bacillus alvei Cheshire & Cheyne
Bacillus bombycis auctt.
Bacillus larvae White
Bacillus laterosporus Laubach (= *Bacillus orpheus* White)
Bacillus lentimorbus Dutky
Bacillus popilliae Dutky

A restudy of the approximately 50 alleged species from which these few were taken would undoubtedly reveal additional valid members of the genus. Since the majority of these organisms are not available in pure cultures, the likelihood of any such study being accomplished in the near future is slight. Woefully inadequate descriptions make it impossible to ascertain the validity of most of them from the literature alone. It is to be hoped that in the future those who describe new species of entomogenous bacteria will avoid misuse of the generic name *Bacillus* and credit their work with complete and pertinent descriptions of the bacteria they name.

The Foulbroods

For centuries it has been known that the honeybee (*Apis mellifera* Linn.) is subject to diseases of one sort or another. Aristotle noticed such disorders in hives under his observation and attributed them to an intoxication by faulty nectar or pollen. He also states, in his "Historia animalium," that bees "suffer most from diseases when the woods produce flowers infected with rust, and in dry seasons." Pliny made similar observations and concurred with Aristotle as to the cause of the maladies. In all probability beekeepers continued to notice such abnormalities in their hives on up to more modern times. In 1586 the famous German apiculturist Nickel Jacob not only described certain bee diseases, which he believed had their origin in putrefactions, but also suggested methods of combating them. In the years to follow, other European beekeepers became interested in and joined in the discussion of the various afflictions to which bees were subject (see Toumanoff, 1930). It is almost impossible, however, to determine with any degree of accuracy just which of the diseases of bees, as we know them today, were the concern of these early

observers. Undoubtedly some of them were dealing with the conditions that today are generally considered under the name "foulbrood."

Among the first to use the name "foulbrood" (analogous to the French designation *la loque*) was Schirach (1771), who, in actuality, apparently used the term to refer to more than one abnormality of bees. It was probably Dzierzon (1882) who first clearly recognized that there were at least two kinds of foulbrood. A similar belief was expressed by Cheshire in 1884, but he later concluded that there was but one. The latter view was for a while fortified by the findings of Cheshire and Cheyne (1885), but during the decade from 1890 to 1900 many American beekeepers began to realize that at least two different diseases were being referred to by the name "foulbrood." This became an established fact with the appearance of the publications of White (1920*a*,*b*,*c*), whose work is still a brilliant chapter in the history of apiculture as well as that of insect pathology.

Even today the term "foulbrood" by itself frequently is used in a general sense, meaning any one of several diseases that attack bees. Usually, however, a condition is meant in which the brood is attacked by bacteria, as differentiated from the fungous, virus, and protozoan diseases. Most authorities generally recognized three distinct types of foulbrood, each caused by a different species of bacterium: American foulbrood, European foulbrood, and parafoulbrood. Let us now consider each of these maladies in some detail.

American Foulbrood

In various parts of the United States, American foulbrood is also known by the common names "black brood," "ropy brood," "diseased brood," "foulbrood," or simply as "foul." It is unfortunate that the word "American" was used in naming the disease, since it occurs in other parts of the world as well as in the United States. European literature refers to it by such names as "*Brutpest*," "*Faulbrut*," "*Bipest*," "*loque américaine*," and the like. Regardless of the name applied, the disease of the brood of the honeybee we are concerned with here is that caused by the specific bacterium *Bacillus larvae* White.

As just indicated, American foulbrood occurs in bees throughout most of Europe, and in Australia, New Zealand, Canada, Cuba, and elsewhere. In the United States it is known to occur in every state in the Union. In some sections of the country its spread has been restricted, but no large beekeeping area is entirely free of it.

American foulbrood has been recognized as a single distinct disease of bees since about 1900. Prior to that time it was confused with European foulbrood and other brood diseases. Throughout the history of beekeeping

American foulbrood has undoubtedly caused tremendous annual losses to the apiculturist. An exact estimate of this loss is difficult to render, but it has been estimated that from 5 to 10 per cent of the colonies in the United States harbor the causative bacterium of American foulbrood. Today, with modern measures of sanitation fairly well understood by most beekeepers, and with known effective methods of treatment, it is largely through negligence that a modern apiculturist need suffer continuing great loss as a result of this disease. This is not to say that American foulbrood is not a serious disease—it definitely is. Not only does it kill large numbers of individual bees; it destroys colonies. Of all the brood diseases it is the one most feared by beekeepers, since it is always a potential menace ready to wreak destruction whenever protective measures are slackened.

Symptoms of American Foulbrood. Before considering the symptoms and pathology of American foulbrood in the honeybee, it may be well for the student who is not familiar with the appearance of healthy brood to consult some description of the immature stages of the insect, such as that given by White (1920*b*). This will enable him to make the comparisons necessary for a thorough understanding of the various changes that occur in the diseased brood.

The first sign of infection is usually a slight brownish discoloration of the normally white larva. The insect loses its normally well-rounded appearance and gradually sinks down in the cell until the posterior end rests against the lower side of the cell. During this time the larva becomes darker in color until it is dark brown or of a chocolate shade. As the insect dries down it becomes a mahogany brown and reaches a very dark shade as it approaches the dried "scale" stage, in which it adheres tenaciously to the cell wall. Infected larvae usually reach maturity, and the cells of the comb are capped. In most cases, death occurs in the capped cells after the larvae have spun their cocoons and are fully extended on the floor of the cells, *i.e.*, in the prepupal stage. Occasionally, a few larvae may die while coiled on the bottom of the cells. Sometimes death comes after the pupa has formed but before the body, except the eyes, is pigmented. The dead pupae resemble in color and consistency larvae dead of the disease. They dry down to a scale residue. The mouthparts of the dead pupa may protrude from the head of the scale and appear as a fine thread slanting slightly backward into the cell, and at times adhering to the upper wall. This protruding part is commonly spoken of as the "tongue" of the pupa and is a symptom characteristic of American foulbrood.

The caps over the cells containing infected brood are usually sunken, discolored, and perforated, and such cells may be scattered throughout

the brood area of the comb, giving it a "pepperbox" appearance. As someone has expressed it, apparently the adult bees are inquisitive to learn why certain sealed cells are not giving forth adult bees; consequently they gnaw a tiny hole in the cap, this being all that is necessary for them to ascertain the trouble. The sinking of the caps is caused by the viscid

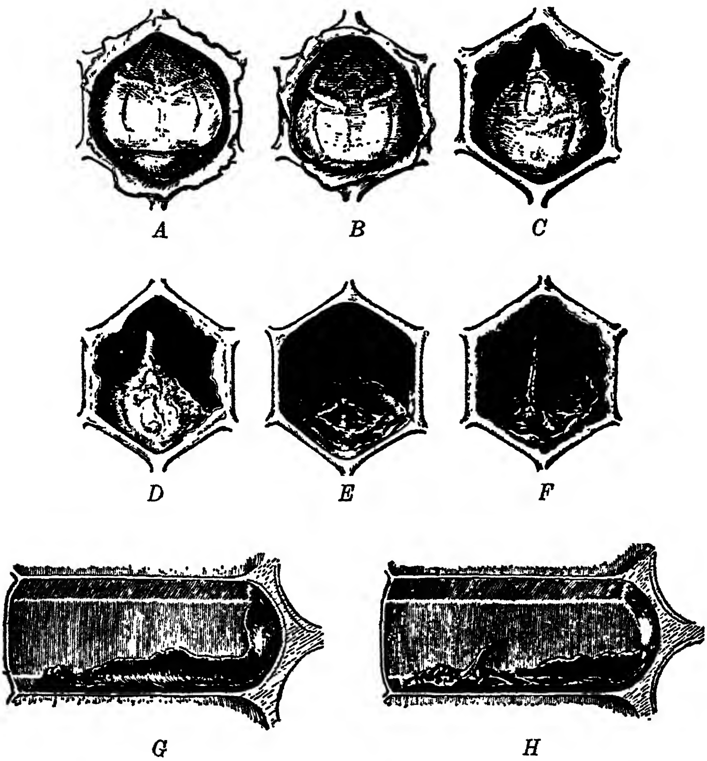


Fig. 67. Larva and pupa of the honeybee, *Apis mellifera* Linn., showing certain of the symptoms of American foulbrood. *A*. Healthy pupa. *B–F*. Stages in the decay and drying of pupae. *G*. Scale of dead larva, lateral view. *H*. Scale of dead pupa, lateral view. (*From Burnside and Sturtevant, 1936.*)

decaying mass within the cell which, adhering to the cap, tends to draw the latter inward as it settles.

Assisting one in diagnosing the disease is the characteristic gluepot or burned-glue odor of brood that have been diseased or dead for some time. Of equal help is the fact that when a toothpick, pointed match, or other slender object is inserted into the mass contained within a cell and withdrawn, the remains of the diseased larvae will adhere to the object, stringing out in a thread of gummy substance for a considerable distance (Fig.

68). This material usually has a ropy consistency. Also of diagnostic value is the Holst test, which is specific for American foulbrood. This test consists essentially of placing the suspected material, *e.g.*, a dried scale, into diluted warm milk. If spores of the causative agent of American foulbrood are present, the milk will curdle and clear in from 5 to 10 minutes. This liquefaction of the milk is brought about through the action of an enzyme produced when the spores of the bacillus are formed. Under ordinary conditions the enzymes persist in the scales for years.

Fig. 68. Ropy, gummy consistency of the remains of a honeybee larva dead of American foulbrood. *(Courtesy of C. E. Burnside.)*

In addition to the symptoms observable in the individual insects, changes may also be noted in the colony as a whole. If recently infected, the strength of the colony may not be noticeably affected. On the other hand, if the infection has been present for a considerable period of time, the colony will usually be weakened. If the disease continues unabated the colony may die. American foulbrood is a persistent disease, and once a colony is infected it seldom recovers of its own accord.

Besides distinguishing American foulbrood from other infectious conditions, it is occasionally necessary to be sure that certain noninfectious conditions are not involved. Addled brood of bees, for example, might be mistaken for American foulbrood by the similar appearance of the cappings over the dead brood. Addled brood usually die in the pupal or prepupal stage. The larvae appear as though they had not been completely nourished and, having failed to reach maturity, had died and undergone autolysis. Pupae and bees almost ready to emerge from their cells may also be found dead. The superficial cause of addled brood is a defective queen; the basic cause has not been determined with certainty (Tarr, 1937*b*).

The Exciting Cause. As with the microbial diseases of most animals, those of bees were originally thought to be due to any of a number of miasmic causes, vapors, putrefactions, poisons, and the like. For example, in an old book on bees written in 1682 by a schoolmaster named Gandernackee, we find:

The bees have a terrible disease which is called foulbrood. It smells terrible and is the right plague . . . . it comes from the following causes: If there is some

place a dead dog lieing upon which the bees fly in spring to collect substances from which they nurse the brood, this is where the poison comes from. Every dead dog should be buried on account of the bees.

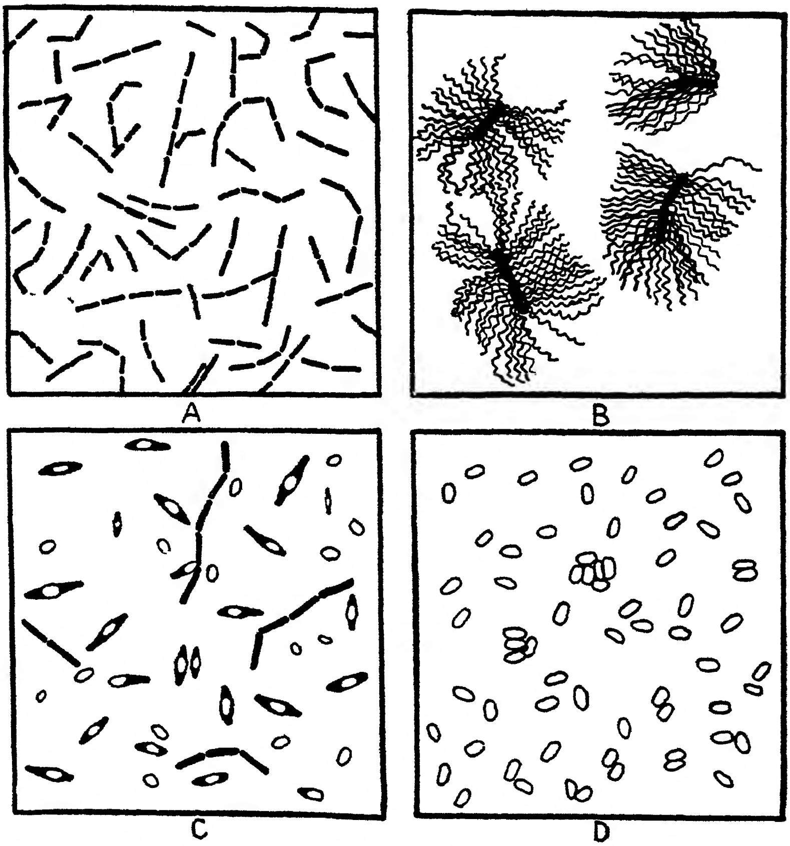


Fig. 69. Drawings of *Bacillus larvae* White, the cause of American foulbrood. *A.* Vegetative rods. *B.* Vegetative rods showing arrangement of flagella as seen after applying a flagella stain. *C.* A mixture of vegetative rods, rods containing spores, and free spores. *D.* Free spores, fully formed. The resistant stage of the bacillus. (*Redrawn from White, 1920a.*)

Until 1904, when White reported the isolation of "Bacillus X" in larvae suffering from American foulbrood, the true cause of this disease was commonly confused with that of European foulbrood, *Bacillus alvei* Ches. & Chey. Shortly thereafter, White (1905, 1906) named the organism *Bacillus larvae.* The names *Bacillus brandenburgiensis* Maassen and *Bacillus burrii* Cowan were proposed by other authors subsequent to this, and the name proposed by White is generally conceded to have priority.

Bacillus larvae White is a gram-positive, motile, sporeforming, rather pleomorphic, rod-shaped organism occurring singly and in chains. When grown on solid media its average size is approximately 2.5 to 5 microns long by 0.5 to 0.8 micron wide; in liquid media it is usually of greater length, tending to form filaments. In carbohydrate media, the bacillus ferments dextrose, levulose, galactose, salicin, and xylose with the production of acid but no gas. With some strains a slight amount of acid is produced from lactose and sucrose. Mannite and dulcite are not fermented. Some strains liquefy gelatin slowly, and the liberation of the proteolytic enzymes is thought to occur concomitantly with sporulation. Starch is not hydrolyzed, nor is indole formed from tryptophane. Slight amounts of ammonia and hydrogen sulfide are produced in the appropriate media. Nitrates are reduced to nitrites; in fact, the bacillus is able to produce nitrite in carrot or turnip media with no added nitrate (Lochhead, 1937). The optimum hydrogen-ion concentration for growth lies in a pH range between 6.5 and 7.0. The optimum temperature for growth has been reported to be about 37°C. The bacillus is a facultative anaerobe.

The spores of *B. larvae* are very resistant to most environmental extremes. They remain alive and virulent for years in the dry remains of larvae and pupae in dry soil, and in old cultures. White (1920*a*) found that a considerable variation exists in the resistance of spores to heat. Many spores are killed within 1 minute at 100°C., and, for some samples, all of them are killed in less than 5 minutes. The most resistant spores, suspended in water, appear to be unable to withstand 100°C. for 11 minutes. They withstand more heating when suspended in honey. According to Burnside (1945), spores of *B. larvae* normally capable of germinating soonest are probably the most highly virulent but are the first to be destroyed by heating. Spores that normally require prolonged incubation to germinate are highly resistant to heating but probably are not virulent. Boiling for 30 minutes can be depended upon to destroy the virulence of the spores under any ordinary conditions. When dry, they are destroyed by the direct rays of the sun in from 28 to 41 hours, although when they are suspended in honey 4 to 6 weeks may be required to kill them. At room temperature, the spores are able to resist 5 per cent phenol for months; 1:1,000 mercuric chloride for days; 10 per cent formalin for hours; and 20 per cent formalin for minutes. The destructive effects of fermentation are resisted for at least 7 weeks, and probably for much longer.

Soon after White first successfully cultivated *B. larvae*, which does not grow on ordinary laboratory media, on an artificial medium (in this case a mixture using bee larvae), subsequent investigators began searching for a medium on which the organism could be grown with ease. White soon

found an unheated egg-yolk agar to be a more suitable medium than the bee-larvae agar. Sturtevant (1924) used a medium in which sterile egg yolk was added to a yeast-peptone base. Lochhead (1928, 1933), in Canada, discovered that plant extracts were useful in cultivating the bacillus and that media containing carrot or turnip extract in addition to peptone and yeast enable the organism to develop satisfactorily. Minced tissues of the developing chicken embryo were used as a substratum by Tarr (1938) for bringing about the germination of the spores of the bacillus. Stoilowa (1938) reported satisfactory growth on a glucose-blood-agar. Holst and Sturtevant (1940) used a yeast-peptone-glucose medium to which they added carrot extract and cysteine. In 1942, Lochhead clarified matters considerably by discovering that thiamin completely replaced the growth-factor effect of such addenda as vegetable extract, yeast, or egg yolk. The bacillus grew well on a medium containing salts-sugar solution, peptone, and thiamin. In certain media, at least, the amino acid histidine appears to be essential for the organism's growth. One of the most efficient easily prepared media for general use is still the peptone–yeast–carrot-extract combination, and a semisolid medium appears to permit more rapid germination of spores than does a solid medium containing these ingredients. The incorporation of glucose in media noticeably decreases the longevity of the bacillus and suppresses spore formation (Katznelson and Lochhead, 1944). Pollen extract included in certain media appears to enhance sporulation (Smith, *et al.*, 1949).

Of interest is the finding by Holst (1945) that *B. larvae* produces, at the time of sporulation, an antibiotic capable of inhibiting the growth of both gram-negative and gram-positive bacteria including certain acid-fast species. The antibiotic is soluble in water but not in organic solvents or alcohols; possesses moderate heat stability and duration of potency; and is greatly inhibited by the presence of glucose but not of sucrose, glycerol, xylose, or cysteine. Although somewhat toxic when injected intraperitoneally into mice, it is not toxic when administered orally.

Predisposing Causes. The exciting cause of American foulbrood, *B. larvae*, may depend upon certain contributing factors, or predisposing causes, in order to bring about a frank infection in the honeybee. Age is one such factor, since infection takes place only during the feeding stage of the larva, with death usually occurring after the feeding stage is past. It has been shown experimentally that larvae are most susceptible during the first 24 hours following hatching, and that larvae 2 days old or older are not susceptible. The disease does not kill older pupae, and adults do not become infected. Sex does not seem to be an important predisposing cause, since worker, drone, and queen larvae are all susceptible.

Complete immunity to the disease is shown by no race of bees. All

strains of bees commonly found in American apiaries are susceptible to American foulbrood, although all are probably not equally susceptible. Claims to success in producing resistant strains of bees through breeding and selection have been made, but these are open to various interpretations as to the true nature of the resistance or immunity involved.

Climatic and seasonal changes do not seem to affect the susceptibility of the larvae to any appreciable extent. It is true that most of the losses from American foulbrood occur later in the bee season than do those from European foulbrood and sacbrood, but this is due more to environmental conditions existing at the different seasons than to any real difference in susceptibility. Brood may be experimentally infected at any season of the year. It should be remembered that the disease may work slowly in destroying the colony. A colony may become infected during the early spring, the disease increasing slowly through the summer, but with the colony still appearing fairly strong by fall. During the winter, however, such a colony usually dies. On the other hand, newly infected colonies may show symptoms of the disease very early as well as late in the season. The appearance of the disease to a large extent depends upon the time when the diseased honey is used for rearing brood. Sometimes small amounts of infected honey are stored in the bottom of cells and later covered up with nectar or sugar sirup. Since such cells may not be emptied for some months, the appearance of the disease will be delayed. Usually, however, if robbing has occurred, the disease appears during the first season, since the contaminated honey is ordinarily stored where it will be soon used for rearing purposes.

To some extent food may constitute a contributing factor to the disease. White (1920*a*) decided that the infection probably is governed somewhat by the quantity of food present and to a less degree, if at all, by its quality. If Sturtevant's (1924) observations are correct, the quality or constitution of the food may have some significance in the disease. According to this worker, the food of the older honeybee larva contains a high percentage of reducing sugar, which is derived from the honey or nectar used in its production. The concentration of reducing sugar in the larval intestine is more than sufficient to inhibit the growth of *B. larvae* until after feeding has ceased. After feeding ends, the remaining reducing sugar is so rapidly assimilated that by the seventh day the concentration of sugar has been reduced sufficiently for active growth of the bacteria to occur. The appearance of the disease in the late larval or early pupal stages is thus explained. A somewhat contradictory set of data has been gathered by Tarr (1938), who noticed that germination of the spores and multiplication of the vegetative cells of *B. larvae* took place in the presence of concentrations of reducing sugars as high as 12.5 per cent in a chicken-embryo

medium. Sturtevant found that concentrations of glucose as low as 2 or 3 per cent inhibited the organisms. This point needs further investigation.

Pathogenesis and Pathology. In spite of the great amount of work that has been accomplished on American foulbrood, only meager in-

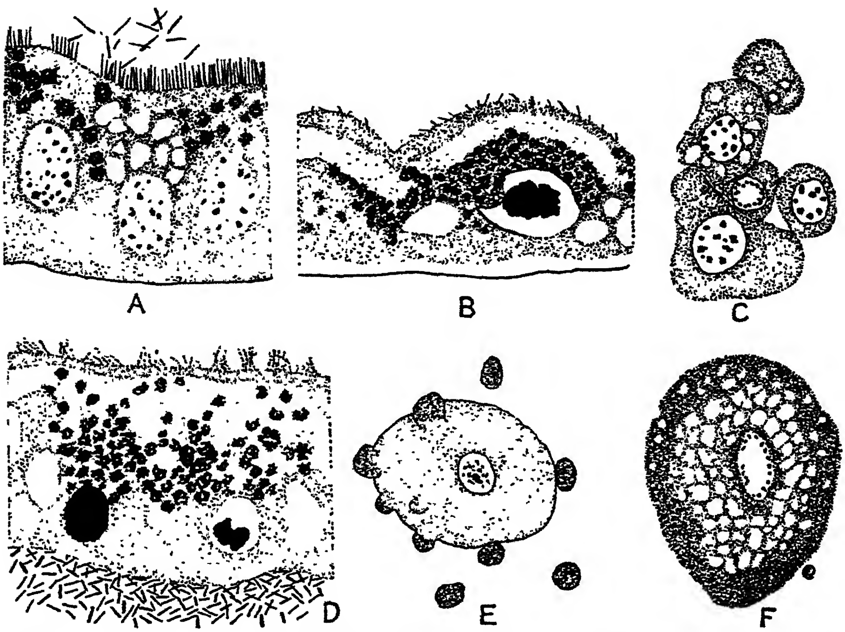


Fig. 70. Certain of the pathological changes seen in the tissues of honeybee larvae suffering from American foulbrood. *A*. Midgut epithelium from larva with a light infection. Note formation of vacuoles and "plasma clumps." *B*. Degeneration of epithelium of Malpighian tubes. Nucleus in pycnotic degeneration. *C*. Fat cells of an infected 3-day larva, only slightly filled with fat globules. Nuclei larger than normal. *D*. Midgut epithelium of an infected 3-day larva, showing degeneration of the cytoplasm and pycnotic nuclei. Cell membranes have disappeared. Note vacuoles, "plasma clumps," and destruction of the striated border. Rods at bottom of figure represent the bacteria in the blood of the insect. *E*. Oenocyte from a 6-day infected larva, with droplike excretions, more darkly colored than the parent cytoplasm. Cytoplasm becoming granulated and nucleus in chromatic degeneration. *F*. Oenocyte of an infected pupa. Note vacuolization, darker periphery of cell, and chromatolysis of nucleus. (*Redrawn from Jaeckel, 1930.*)

formation is available concerning its pathogenesis, or course of development, in the honeybee.

On the basis of histological sections, Maassen, in 1908, affirmed that the causative bacillus does not come to luxuriant development in the intestine of the larva but that it finds more promising nourishment in the fat body. That the alimentary tract does not appear to be initially

involved has been confirmed by subsequent observers. Jaeckel (1930) decided that the bacillus penetrates the epithelial lining of the insect's gut and produces a type of septicemia. The blood carries the organism to the various organs of the body and the infection proceeds until, after all body tissues are invaded (although actual development of *B. larvae* does not take place in the fat tissue), the insect dies and the characteristic ropiness and decay of the brood sets in. The tissue cells and their nuclei undergo degeneration and then dissolution. Phagocytosis does not serve as much of a defense against the bacillus. Jaeckel has described the histopathological changes in several of the affected tissues, particularly the Malpighian tubes and midgut epithelia, the fat cells, and the oenocytes (Fig. 70). This differs somewhat from the situation in European foulbrood which, at least to begin with, is essentially an intestinal infection. In some instances of American foulbrood, pathological changes in the cells of certain tissues take place, although the bacteria are not in the immediate vicinity—as though some sort of toxic effect may, to some extent, be present. (See also Tarr, 1937*a*, page 168.)

Transmission of *Bacillus larvae*. The portal of entry for *B. larvae* appears to be somewhere along the alimentary tract of the honeybee larva. This is evidenced by the fact that the disease results after the insect is fed food contaminated with the bacillus. It is logical to assume, therefore, that one of the principal methods by which the bacillus is transmitted in nature is through the food and probably through the water supply. The tendency of bees from healthy colonies to rob the stocks of diseased and weakened colonies is probably the most likely method by which the organism is transmitted in nature. There is good evidence that wild bees are a source of American foulbrood. The placing of brood combs containing diseased brood with healthy colonies may also provide for the transmission of the disease. Flowers visited by bees, the clothing and hands of the apiarist, and beekeeping tools and supplies were considered by White to be unlikely sources of infection.

Control of American Foulbrood. Attempts to control American foulbrood, *i.e.*, attempts to eradicate it or to keep it from spreading, may be considered as being of any of four types: production of resistant strains of honeybees, the institution of sanitary control measures, chemotherapy, and legal regulations.

In 1945 the U.S. Department of Agriculture officially stated that definite progress had been made in their attempts, through breeding and selection, to produce a strain of bees resistant to American foulbrood. This statement was followed by other similar reports made by certain State Agricultural Experiment Stations, notably those of Iowa and Texas. During the years prior to these reports, practical beekeepers had observed

that certain colonies of bees appeared capable of contending with the disease more successfully than others. The exact nature of this resistance, however, has still not been generally agreed on. Woodrow and Holst (1942) found that disease could be produced in the brood of resistant colonies as readily as in that from susceptible ones. All diseased brood were removed by the bees of some resistant colonies before ordinary symptoms of the disease were evident. In a resistant colony no diseased brood remained long enough to permit the disease organisms to reach the spore stage, whereas in a susceptible colony spore formation occurred in numerous infected larvae. *Bacillus larvae* in the vegetative or rod stage is noninfectious. The data obtained by Woodrow and Holst showed that resistance to American foulbrood in the honeybee colony consists in its ability to detect and remove diseased brood before the causative organism reaches the infectious spore stage in the diseased larvae. This reasoning was supported by the findings of Filmer (1943), who concluded that the resistance of some strains of bees to American foulbrood is not a true immunity but is due to certain housecleaning characteristics of the bees. And, in line with the more recent results obtained by the U.S.D.A., Filmer suggests that further selection and breeding are necessary to produce a strain of bees satisfactorily resistant to *B. larvae*. In the meantime the use of the resistant stocks available in conjunction with other remedial measures would seem to be advisable.

It is obvious that truly resistant strains of honeybees would be the most desirable solution to the problem of controlling American foulbrood. In the absence of this ideal remedy, however, beekeepers have for years employed stopgap remedies of all variations in kind and effectiveness. The safest and quickest means of eradicating the disease in an apiary is to burn the diseased colonies after first killing the bees by placing a tablespoon of calcium cyanide in the entrance and into the top of the hive. To decrease the danger of interference by robber bees, the killing of the bees and the burning should be done at night. Hambleton (1933) advises that the material to be burned should be placed in a pit 18 inches or more deep, and after everything is burned the pit should be filled again with soil. In most instances, the bottom board, hive bodies, inner covers, and tops may be saved, in which case these materials should be thoroughly scraped, and scrubbed with a hot soap or lye solution. The hive bodies may be sprinkled with kerosene or gasoline and ignited so as to scorch the insides effectively. This may also be accomplished with a blowtorch.

Although burning the infected colonies may be relatively inexpensive in the long run, beekeepers have always been reluctant to destroy their combs, which may represent a considerable investment. Accordingly, methods of by-passing this rather drastic procedure were sought. Some

early recommendations, such as the shaking method by which healthy bees in a diseased colony are shaken from the old combs into a clean hive on clean frames, were found to be inadequate and dangerous and had to be rescinded. Disinfecting solutions are of only limited value in the treatment of American foulbrood, since none have been found that will thoroughly sterilize the bee-containing combs or the spores in sealed honey without destroying the comb or poisoning the honey. Supercombs that have never contained brood may be disinfected effectively with a 20 per cent formalin-water or formalin-alcohol solution. The combs should be kept immersed in such a solution for at least 24 hours.

Even before White, in 1920, published a list of drugs (*e.g.*, phenol, formic acid, quinine) he had employed in an effort to effect a control for American foulbrood, European beekeepers had used medicated sirup for the same purpose. None of the early attempts, however, yielded much of practical value. Because of the apparent uselessness of such methods, little effort was spent in experimentation along this line in subsequent years except that between 1928 and 1942 such substances as iodine, thymol, and the whey from cheese were advocated for such use. Then in 1942 Haseman and Childers (1944), inspired by the advent of the sulfa drugs in the treatment of human infections, added sulfanilamide to sugar sirup and fed it to foulbrood-infected bees with highly promising results. They extended their trials to the use of sulfadiazine, sulfaguanidine, and sulfathiazole, with particular emphasis on the latter drug, which was recommended (Haseman, 1946) as an effective control for the disease when fed either in sugar sirup or in pollen substitute. One-half gram of sulfathiazole per gallon of sugar sirup was the recommended dosage. Tests similar to these were being conducted in England with sulfapyridine (Milne, 1945). In addition to the sirup-feeding method, successful treatment has been reported (Latham, 1947) using alcohol-dissolved sulfathiazole as a spray applied directly on the infected combs.

Although such antibiotics as penicillin and streptomycin have been given preliminary trials, there is very little evidence to indicate their practicability under average beekeeping conditions. Johnson (1947) reports that penicillin buffered with calcium carbonate, used at the rate of 50,000 units to 1 quart of sugar sirup at weekly intervals, is not effective in controlling the disease in a populated hive. He also reported essentially negative results with furacin (5-nitro, 2-furaldehyde semicarbazone) with X-ray treatments, and with sulfapyridine. As did his predecessors, Johnson observed that sulfaguanadine and sodium sulfathiazole used at the rate of 0.5 gram to 1 gallon of water were promising means of treatment. Doses of 1 and 2 grams of sulfathiazole per gallon of sugar sirup are not appreciably more efficient in eliminating the disease

than the recommended dosage of 0.5 gram per gallon of sugar sirup. According to Haseman (1948), however, in the laboratory penicillin and streptomycin are more effective in inhibiting the vegetative growth of *Bacillus larvae* than are the sulfa drugs.

The factors involved in the action of sulfathiazole on colonies infected with *B. larvae* have received only meager consideration. The basic or fundamental action is undoubtedly the bacteriostatic one already well known in the case of the sulfa drugs when used against other animal and human pathogens. Reinhardt (1947) has explained the over-all effectiveness of sulfathiazole on the observation that bee colonies fed sugar sirups, with or without drugs, are stimulated to remove dead brood more effectively than do unfed colonies; the bacteriostatic or bactericidal action of the drug suppresses the disease, giving the bees an opportunity to remove the diseased bees fast enough to overtake the infection, and the hive is thus cleaned up. Permanent cure of the disease requires that infective material within the hive be removed or consumed while the drug is being fed and is effective in preventing brood mortality.

Beekeepers have been quick to grasp the sulfa-drug method of control. Widespread use of sulfathiazole has been made, and there is every reason to believe that, if used wisely, it may have a definite place in the control of American foulbrood. Indiscriminate use of the drug, however, is not without its harmful consequences. This has been pointed out in the cautious reports of several experimenters, including Hambleton (1947), Lesher (1947), and Eckert (1947*a*).[1] Not only may careless use of the sulfa drugs produce sulfa-resistant strains of *Bacillus larvae*, but it may perpetuate colonies highly susceptible to the disease, thereby nullifying much of the work already accomplished on resistant stocks. The apparent disappearance of the infection may lead the careless observer to believe that it has been completely eliminated, and this false sense of security may result in the spreading of the bacteria by the interchange of contaminated combs. The application and use of the drugs should be conducted under the supervision of qualified inspectors. In no sense does its use lessen the need for periodic inspection on the part of the beekeeper or of the inspection service. Since some persons are allergic to even small amounts of sulfa drugs, the fact that the drugs are deposited in the honey of treated hives makes their use of concern from the public-health standpoint.

The effective control of American foulbrood, as well as that of other bee

[1] The latter worker (Eckert, 1948) has concluded that the general use of sulfathiazole as a *preventive* measure in the control of the disease is not justified at the present time because of the danger inherent in introducing even small quantities of the drug into marketable honey.

diseases, has necessitated the establishment of certain laws, legal regulations, and inspection services throughout all the major beekeeping areas of the world. In the United States each of the beekeeping states has apiary-inspection laws and appoints one or more bee inspectors who, among other things, inspect the apiaries for the presence of disease and see to it that infected colonies are properly disposed of and that quarantine measures are instituted where necessary. The laws and regulations vary from state to state, but in general they concern themselves with procedures used by the state apiarist or bee inspector in carrying out his work, and with his rights and duties in dealing with diseased hives. The necessity and great value of efficient bee inspection are obvious. In spite of the modern refinement in the treatment of bee diseases, bee-inspection service remains one of the most practical factors in the control of these diseases.

European Foulbrood

Although at first confused with American foulbrood, European foulbrood is now recognized as a distinct disease of the honeybee, *Apis mellifera* Linn., and is known to occur throughout the world, including the United States. The unfortunate designation "European" is no indication of the limits of its distribution and was applied to the disease because of the early work of European investigators. The disease has also been known by the names "New York bee disease" and "black brood," but these terms have been abandoned. It is still referred to as "melting brood," which indicates the condition in which the dead honeybee larva "melts" away from its tracheal system.

European foulbrood is not considered to be so dangerous as American foulbrood, although under certain conditions it can spread through a colony with amazing rapidity, resulting in serious losses of brood. In severe cases the colony may be killed. In general, the tendency of a colony to recover is greater in European foulbrood than in American foulbrood.

Symptoms of European Foulbrood. In mild cases and in the early stages of European foulbrood, the arrangement of the brood in the combs is not noticeably irregular. The degree of irregularity increases, however, with the severity of the disease and the length of time it has been present. When the disease is well established, the brood nest presents a "pepper-box" appearance, since the capped and uncapped cells are scattered irregularly over the brood frames.

The diseased larvae lose the plumpness and glistening white color of healthy larvae and become flat white. At the time of death, or soon thereafter, they take on a faint yellow color, which later becomes brownish; eventually this deepens to a dark brown. The infected larvae may show

abnormal movements and occupy unnatural positions in the cells. Most of them die while coiled on the bottom of open cells; a few die while fully extended.

After the larvae die their remains undergo decay but do not become characteristically ropy as in American foulbrood. Marked viscidity is ordinarily absent. The tracheae in the dead larvae usually show more clearly than in healthy ones. They stand out in relief as radiating white

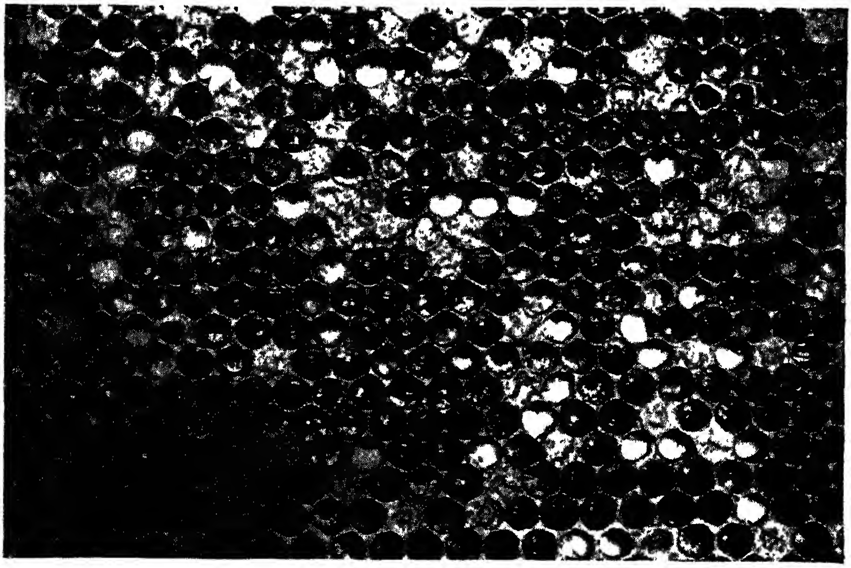


Fig. 71. European foulbrood. Heavily infected comb showing larvae in various stages of disease and decay. (*From Burnside and Sturtevant, 1936.*)

lines in the dead coiled larvae and as narrow white lines across larvae that die while extended. A white line that crosses the radiating white lines can frequently be seen on the side of the larvae. This is a valuable but not an absolutely dependable symptom of European foulbrood (Burnside and Sturtevant, 1936).

When viewed through the dorsal integument of a diseased or recently dead larva, an elongated, dull grayish-white or yellowish-white mass can be seen within the chyle stomach. This mass consists of a turbid fluid containing numerous bacteria. The bright yellow mass seen in this location in healthy larvae consists of pollen.

Immediately after death, and for a short time thereafter, the larvae can be removed from the cells without disrupting the body wall. Within a few days, however, the integument and other tissues become soft, the larvae settle against the lower wall of the cells and appear moist, "melting,"

flattened, somewhat translucent, and they cannot be removed without tearing or disrupting the skin.

As the process of decay subsides, the dead larvae dry down to a dark-brown scale. The color of the scale varies according to whether the larvae die before the cells are sealed, in which case drying takes place rapidly, stopping the decay, and leaving the scales light-colored, or whether they die after the cells are sealed, in which case drying is slow, decay is prolonged, and the scales are dark brown or nearly black. Unlike the scales in American foulbrood, those in European foulbrood do not cling closely to the wall of the cell and are easy to remove.

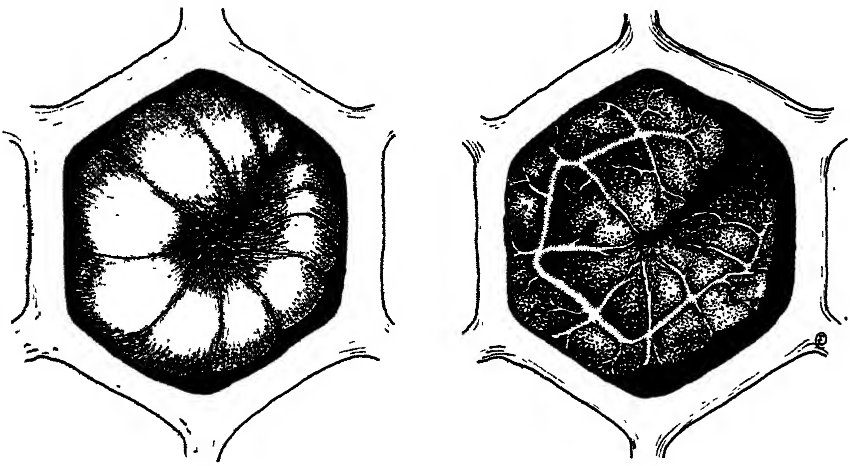


Fig. 72. Honeybee larva dead of European foulbrood. *A*. Normal healthy larva. *B*. Dead larva giving the appearance of "melting" away from the tracheal system.

According to Burnside and Sturtevant (1936), larvae that die of European foulbrood in sealed cells may become quite ropy and resemble larvae dead of American foulbrood. Since the bees remove dead brood from open cells first, it sometimes happens, after the disease ceases to be active, that the brood that died in sealed cells is all that remains in the combs. In such circumstances it may be difficult to determine whether American foulbrood or European foulbrood or both these diseases are present. It should be remembered that some of the changes in dead brood may be brought about by secondary invaders, especially bacteria. This, of course, would tend to vary or to modify the changes seen.

An odor characteristic of the disease can sometimes be detected. It is usually described as a "sour odor," or an odor of spoiled meat.

The symptomatology of European foulbrood is limited largely to the

larvae, since pupae are rarely affected by the disease. As in American foulbrood, adults are not affected.

The prognosis of European foulbrood varies from very good to exceedingly grave. As we have already indicated, the tendency for a colony

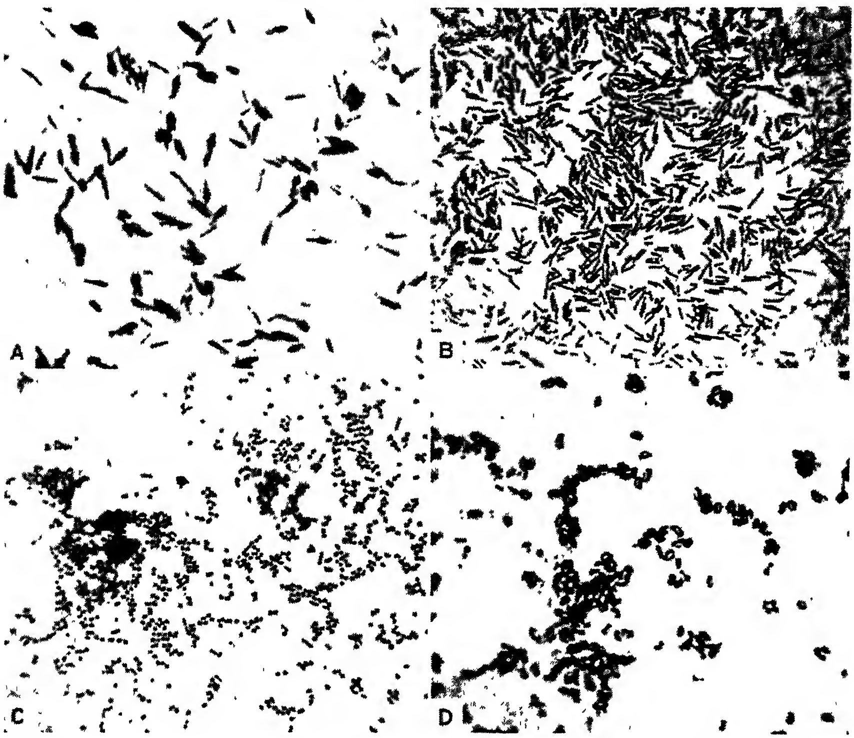


Fig. 73. Some of the bacteria associated with European foulbrood of bees. *A. Bacillus alvei* Ches. & Chey., generally considered to be the form that constitutes the primary cause of the disease. *B.* An asporogenic form of *Bacillus alvei*, morphologically closely resembling White's *Achromobacter eurydice* (White). *C.* "*Bacillus pluton*" White from the stomach of a bee larva in the advanced stage of European foulbrood. *D.* Spores of *Bacillus laterosporus* Laub. (=*B. orpheus* White), one of the occasional secondary invaders found in bee larvae suffering from European foulbrood. (*Photographs courtesy of C. E. Burnside.*)

to recover entirely from the disease is much greater than in the case of American foulbrood.

The Exciting Cause. The identity and taxonomy of the agent responsible for European foulbrood have undergone considerable argument and discussion. Although some writers are still not too careful about this point, it is now generally recognized that the exciting cause of the disease is the sporeforming bacterium *Bacillus alvei* Ches. & Chey. At any rate

it is the organism that has been repeatedly isolated from bee larvae affected with the disease, although its pathogenic properties have not been entirely clarified.

Bacillus alvei was described as the cause of European foulbrood in 1885 by Cheshire and Cheyne. Since then considerable difference of opinion has been expressed concerning the etiology of the disease and the true status of the bacillus. Maassen (1907, 1908) believed that a combination of *Bacillus alvei* and an organism he named *Streptococcus apis* (considered by some to be synonymous with *Streptococcus liquefaciens* Stern.) were necessary to cause the foulbrood. In 1908 White observed a bacterium, referred to as bacillus "Y," which would not grow on the usual artificial media. In 1912 he considered this nonsporulating organism to be the exciting cause of European foulbrood and gave it the name *Bacillus pluton*. White maintained this position in his comprehensive report on this disease in 1920, considering *Bacillus alvei* Ches. & Chey., *Streptococcus apis* Maassen, *Achromobacter* [*Bacterium*] *eurydice* (White), *Bacillus laterosporus* Laub. (*Bacillus orpheus* White) to be secondary invaders.

For several years following the work of White, the etiological role of *Bacillus pluton* White was accepted, with some workers (*e.g.*, Sturtevant, 1925) pointing out that even in a secondary role *Bacillus alvei* probably had a marked influence upon the course of the disease. Then in 1928, Lochhead showed that *B. alvei*, when grown for several weeks on sugar-containing media, possessed a coccoid stage that appeared similar to *B. pluton*. Lochhead questioned whether *B. pluton* as a separate species could be said to exist at all, since it has never been known to be obtained in pure culture, Wharton's (1928) report on its cultivation having been discounted by Lochhead. Wharton states, however, that "cultures of *B. pluton* have been observed to change to *B. alvei* form resembling biologically the *B. alvei* isolated from infected larvae."

In 1934 Burnside published an account of his studies on the bacteria associated with European foulbrood in which he asserted that no evidence has been obtained that satisfactorily explains the etiology of this disease in bees. He noted that several morphologically different bacterial forms are more or less constantly present in honeybee larvae sick or dead of European foulbrood; these forms are absent in larvae sick or dead of other causes. Of particular significance, as regards the present discussion, is Burnside's observation that "*Bacillus alvei* is capable of morphological, cultural, and biological transformation and is also capable of stabilization, at least temporarily, as a sporogenic rod, an asporogenic rod resembling *Bacterium eurydice*, or a coccoid resembling *Bacillus pluton*." As to the identity of *Streptococcus apis* and *Bacillus pluton*, Burnside appears to be quite certain. He further suggests that *Bacillus pluton* and *Streptococ-*

cus apis are variants or stages in the life history of *Bacillus alvei*. Among the several reasons for this belief, he cites the occurrence of variants resembling *Bacillus pluton* in pure cultures of *Bacillus alvei* and the apparent origin on rare occasions of sporogenic *Bacillus alvei* in cultures of *Streptococcus apis*. Tarr (1935) suggests that there may be several distinct strains of *Bacillus alvei* which may be differentiated on the basis of their fermentative powers.

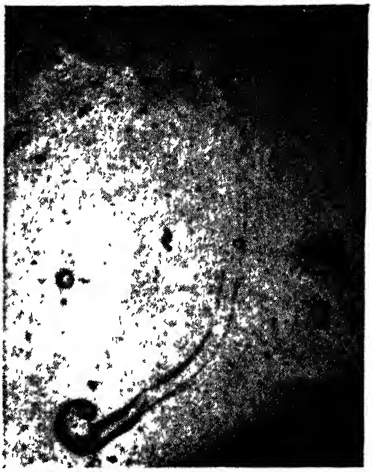


Fig. 74. Photograph of part of a nutrient agar plate showing the creeping phenomenon characteristic of certain colonies of *Bacillus alvei* Ches. & Chey. on the surface of solid media. (*Courtesy of M. Aschner.*)

Several other theories on the etiology of European foulbrood have been presented in the literature, but none of these are based on direct proof. Nearly all recent evidence tends to support Burnside's concept; and, until conclusive data to the contrary are put forward, there seems to be no good reason why *Bacillus alvei* is not a species in good standing and very probably the causative agent of European foulbrood. If further studies establish that *Bacillus pluton* is in fact a separate and distinct species, it should be removed from the genus *Bacillus* since it is not sporogenic.

Neide's (1904) *Bacillus alvei* Krompecher may be considered synonymous with *Bacillus alvei* Ches. & Chey.

Bacillus alvei may be characterized as a gram-variable (usually positive), sporeforming rod, ordinarily exhibiting active motility by means of peritrichous flagella. An interesting characteristic of *B. alvei* is its ability to form motile or migrating colonies upon the surfaces of solid media free from excessive moisture (Smith and Clark, 1938). By the selection of variants, strains that produce nonmotile colonies may be selected. According to Clark (1939), nonmotile colonies do not show the presence of motile organisms, while active motility is exhibited by cells from motile colonies. Nonmotile cells are distinctly capsulated, although this characteristic is lacking in the case of motile strains. A characteristic of motile and nonmotile strains alike is that in suitably aged cultures long rows of cylindrical spores, with the long axes of the spores lying parallel, may be observed. Some authorities consider this property to be almost diagnostic of the species. *B. alvei* is cultivable on numerous artificial media without any difficulty. It requires thiamine for growth, and glycine, leucine, and

cystine are essential or stimulatory, depending on the strain (Katznelson and Lochhead, 1947).

The bacillus is not known to be pathogenic for any insects other than bees, and laboratory animals and man are not susceptible.

During the course of his studies on European foulbrood, White (1920*b*) made numerous determinations of the properties of the causative bacillus. Although he believed *Bacillus pluton* to be the true cause of the disease, his findings in all probability apply to at least the vegetative stage *B. alvei* as well, since the former is now considered to be a nonsporeforming stage of the latter. White's findings may be summarized as follows:

The thermal death point of *B. pluton* suspended in water is approximately 63°C. maintained for 10 minutes. When suspended in honey the bacillus is destroyed in 10 minutes at approximately 79°C. It remains alive and virulent for approximately 1 year when dried at "room or incubator temperatures." It resists the killing action of the direct rays of the sun for from 21 to 31 hours when dry, for 5 to 6 hours when suspended in water, and for 3 to 4 hours when suspended in honey. In the presence of fermentative processes in a 10 per cent sugar solution, *B. pluton* is destroyed in from 3 to 5 days at "incubator temperature" and in from 11 to 21 days at "room temperature." In a fermenting honey solution outdoors, the bacillus retains its virulence for at least 1 month. Putrefactive processes at "incubator temperature" destroy the organism in from 7 to 13 days, at "room temperature" in from 21 to 35 days, and at "outdoor temperatures" it retains its vitality for at least 40 days. In honey at "room temperature," *B. pluton* ceased to be virulent in from 3 to 7 months. Mixed with pollen, the bacteria remained alive and virulent for more than 7 months at "room temperature" and for more than 10 months at "refrigerator temperature." In 0.5 per cent carbolic acid solution, the bacillus is destroyed in from 8 to 18 days; in 1 per cent solution it is destroyed in from 5 hours to 4 days; and in 2 and 4 per cent in less than 6 hours.

Predisposing Causes. The age and stage of the honeybee larva are important factors in determining the susceptibility of the insect to *Bacillus alvei*. According to White (1920*b*), infection takes place during the feeding stage and at some time after the first day of larval life, the larva being more often 2 days of age, or older. Death takes place slightly more than 2 days from the time of infection. Ordinarily, therefore, a larva has passed its fourth day of larval life before death from European foulbrood occurs, and death may occur any time up to pupation. Death as pupae is rare, and adults are not susceptible to the pathogenic action of the bacillus. Sex is apparently of small consequence as far as susceptibility

is concerned, since worker, drone, and queen larvae are all susceptible to the infection.

No race of bees is known to be completely immune to European foulbrood. Caucasian and Carniolan races appear to be less seriously affected by the disease than are common-black bees. Furthermore, the common-black bees and the Italian-black hybrid bees are more frequently afflicted than are pure Italians. The disease often appears year after year in colonies of black or hybrid bees, while among Italian bees losses are usually unimportant, although exceptional outbreaks do occur. Regardless of the race, weak colonies are usually more seriously affected than are strong ones.

There appears to be a noticeable relation between the climate and the occurrence of European foulbrood. Although brood is susceptible at all seasons of the year, the disease is somewhat synchronized with the seasons, being most common in the spring when brood rearing is at its height. The earliest reared brood usually is not affected. Ordinarily the disease subsides by midsummer, although occasionally it continues to be active during summer and fall; or it may reappear in the fall. Sometimes the disease appears suddenly and spreads rapidly within infected colonies. At other times it spreads slowly and does little damage. A good honey flow seems to hasten recovery.

Pathogenesis and Pathology. After the susceptible larva has ingested the causative bacillus the latter multiplies and proceeds with its development within the insect's alimentary tract. According to White (1920*b*), and assuming that his *B. pluton* is but a form of *B. alvei*, the bacteria grow close to the surface of the peritrophic membrane in contact with the food of the larva. As growth continues, the bacterial mass extends toward the center of the lumen of the peritrophic sac, eventually filling it more or less completely. The growth does not always take place uniformly along the peritrophic membrane, nor do the bacteria extend beyond it, but are enclosed within the sac, and the tissues of the host are not reached. These observations have been confirmed by Tarr (1938*b*) and others. In White's opinion, the multiplication of the organism after the death of the host is limited, if, indeed, it takes place at all. Death is caused apparently by toxic products of the bacillus, which diffuse through the intestinal wall to the vital tissues of the insect. The various secondary invaders encountered so regularly in this disease probably also play a role in the insect's destruction.

Transmission of *Bacillus alvei*. Since European foulbrood can be produced experimentally by feeding bees infectious material, it may be assumed that infection takes place by way of the alimentary tract. Any

situation, therefore, that provides for oral contamination is likely to be important from the standpoint of disease transmission. The food and water of the bees are probably the most important carriers of *Bacillus alvei* to healthy bees. Since the bacillus remains virulent in honey for only a few months it is not so important a source of infection as pollen, in which the organisms remain virulent much longer. The tendency of adult bees to remove sick and dead larvae from the brood comb in a piecemeal manner enhances the possibility of contamination.

The principal mode of transmission from one colony to another is probably through the robbing of a diseased colony. When a colony becomes so weakened that it can no longer protect itself adequately, bees from neighboring colonies come in to rob it of its stores; and, if the weakened colony is diseased, the chances that the robber bees will return with contaminated material are great. It is unlikely that the disease is spread by way of flowers visited by bees from healthy colonies that had been visited previously by bees from diseased ones. Nor is it likely that the bacteria are carried in sufficient numbers on the hands or clothing of the beekeeper to initiate new outbreaks. Careless manipulations of the apiary, however, such as placing brood combs from diseased colonies in healthy ones, are an important source of infection.

Control of European Foulbrood. Since the prognosis in most cases of European foulbrood is rather favorable, there has not been so pressing a demand for methods of combating the disease as in the case of American foulbrood. Early experiences showed that a strong colony is essential for the successful control of the disease. The disease has a tendency to subside or disappear entirely during periods of abundant honey flow. The race of honeybee making up the colony may also be important, since it has been shown that the common-black and Italian-black hybrid bees are more susceptible than are the purebred Italians. Therefore, in taking steps to control the disease, the beekeeper should first of all maintain strong colonies by keeping them well supplied with stores and requeening with a vigorous Italian queen.

If the outbreak of European foulbrood is severe, it may become necessary to destroy the colony by burning in the same manner as that which has been described for American foulbrood. Scorching the hive boards, and the use of effective germicides, such as formalin, are useful adjuncts in the treatment of the disease. Sanitary measures and inspection services should be maintained regularly.

Early experiments using medicated sirups to ward off European foulbrood were made by a number of investigators who reported results of varying effectiveness. In general, little if any reliance could be placed in most of the medicaments used. The successful use of sulfa drugs in the

control of American foulbrood has not been duplicated with European foulbrood. Some authors report some curative value with the use of these drugs, while others declare them to be without value. The beekeeper should make certain that the disease is positively identified before specific control measures are initiated.

Parafoulbrood

In 1932 a disease of brood of the honeybee, *Apis mellifera* Linn., was observed in the southeastern part of the United States, which appeared to be different from the brood diseases previously described. To this new disease, Burnside, its discoverer, gave the name "parafoulbrood," and to the causative agent, the name *Bacillus para-alvei.* The next year, a description of the disease and its gross symptoms was presented by Foster and Burnside (1933), and in 1935 Burnside and Foster described the causative organism in detail.

Parafoulbrood is found principally in limited sections of North Carolina, South Carolina, Georgia, and Florida, although it may occur in other areas but remain unrecognized. Losses caused by the disease may vary from the weakening of a few colonies to the loss of entire apiaries. Worker, queen, and drone larvae and sometimes pupae are killed by the disease, but adult bees are not affected. All races of bees common in North America are susceptible, but Italians appear to be more resistant than are common blacks and hybrids. Although heavy losses of brood may take place in strong colonies, the most serious outbreaks occur in weak colonies.

Symptoms. As described by Burnside and Sturtevant (1936), combs from colonies attacked by parafoulbrood resemble combs from colonies suffering from European foulbrood. The brood is more or less irregular. Dead brood in open cells is removed by the bees sooner than that in the sealed cells. Sometimes the bees increase the thickness of the cappings over dead brood in sealed cells. Such cappings appear dark, sunken, and greasy, and are sharply depressed in the center. Dead larvae may remain in these cells for months, or even over winter.

Larvae infected with *Bacillus para-alvei* may become slightly less plump and change in color from a glistening white to a dull or flat white. They move uneasily in their cells and are frequently found in abnormal positions. Just before the larvae die, a yellow discoloration may appear. At the time of death, a large number of the larvae are coiled or irregularly twisted in the cells, although many larvae die when in an extended position. A few may die as pupae. The average age of the larvae at the time of death is usually somewhat greater than in the case of European foulbrood. Furthermore the number of larvae dying in sealed cells is usually somewhat greater, and the number of larvae dying while coiled is less than in European

foulbrood. Larvae dying in open cells dry rapidly and ordinarily form light-colored scales, although some take on darker shades. Those which die in sealed cells dry more slowly. Since decay continues for a longer time in such cells, the larvae become reddish-brown and form dark-colored scales. The scales may be removed easily from the cells. In an occasional decayed larva the tracheae show clearly. The stomach is usually visible through the dorsal integument in sick or recently dead larvae. A turbid grayish or yellowish-gray fluid usually fills the stomach and contains many bacteria.

Fig. 75. Vegetative rods of *Bacillus para-alvei* Burnside, the causative agent of parafoulbrood of bees. (*Courtesy of C. E. Burnside.*)

The consistency of larvae dead of parafoulbrood is usually soft and watery. In capped cells some become decidedly ropy during decay, and form dark reddish-brown or brown scales of a leathery consistency. In open cells, on the other hand, the insects usually become pasty and later form light-colored brittle scales. As Burnside and Sturtevant point out, ropiness in parafoulbrood often resembles this symptom in American foulbrood, but the two diseases can usually be distinguished by noting the color and odor of the dead brood. Recently dead brood have only a slight odor. In sealed cells, and also in some open cells, however, an intense putrid odor develops similar to that of European foulbrood but often much more intense. Therefore, the symptoms of parafoulbrood that reliably characterize the disease include the reddish-brown color and the ropy consistency of decayed brood, expecially when accompanied by a pronounced putrid odor.

From the standpoint of the colony as a whole, the symptoms may vary from a slight weakness of the colony to its complete destruction. In some colonies parafoulbrood progresses slowly, and the disease may disappear of its own accord. In other instances the disease progresses rapidly, seriously weakening or killing the colony. Although the bees of some colonies clean out the dead brood promptly, others allow it to accumulate, endangering the life of the colony. Entire apiaries may be lost through the activities of the disease, but usually the losses are confined to a few colonies.

Causative Agent. The exciting cause of parafoulbrood is generally

considered to be the organism designated by Burnside (1932) as *Bacillus para-alvei*. It is an aerobic, gram-positive, motile, sporeforming rod, extremely variable in size and shape on artificial media. Variations also occur in the insect host where, for example, coccoid forms tapering at one or both ends may be seen in well-advanced cases of the disease. Acid (but no gas) is produced in carbohydrate media. In liquid media, sporulation is retarded, and after 10 or more generations in potato broth, the ability to form spores may be partly or entirely lost, at least temporarily.

It is generally recognized that *Bacillus para-alvei* Burnside is similar in most of its characteristics to *Bacillus alvei* Ches. & Chey. of European foulbrood. Smith, Gordon, and Clark (1946) have presented evidence to indicate that the two organisms are essentially the same species. On the other hand, Tarr (1936) noted that *B. alvei* and *B. para-alvei* differed in the shape of the vegetative cells during sporulation and in the type of endospores produced; and Katznelson and Lochhead (1947) observed certain differences in the nutritional requirements of the two organisms. Such differences as these seem to strengthen the case for separating these bacteria into two species, or at least into two distinct varieties of the same species.

Treatment and Control. Since the behavior of parafoulbrood is so similar to that of European foulbrood, it is generally recommended that the same methods be used for the treatment and control of parafoulbrood as are used for European foulbrood. When more is known about parafoulbrood, perhaps more specific remedial measures will be suggested.

Bacillus Infections of the Silkworm

The silkworm, *Bombyx mori* (Linn.), is subject to bacterial infection just as are most insects. Until recently, one of the important diseases of this insect was ascribed solely to the activities of a sporeforming bacillus which Pasteur, in 1870, designated as the "*vibrion à noyau.*" This disease is best known by the name "flacherie," and the bacterium referred to is called *Bacillus bombycis* auctt. In recent years it has become apparent that the etiology of flacherie is considerably more complex than was supposed by Pasteur and other early investigators of this disease. It is now believed, although further confirmatory proof is needed, that the exciting agent of flacherie actually is an ultravirus, and that *Bacillus bombycis* is a secondary invader. For this reason, our discussion of this bacillus will be postponed until its role in flacherie may be described in detail, along with a consideration of the causative virus. This will be done in Chap. 11.

Other Species. In Japan, in 1902, Ishiwata observed a severe type of dysentery among silkworms and isolated a bacterium which he called the "Sotto bacillus" and which he considered to be the cause of the disease.

TABLE 3. SUMMARY OF SYMPTOMS OF BROOD DISEASES OF BEES*

Symptom	American foulbrood	European foulbrood	Parafoulbrood	Sacbrood†
Causative organism	*Bacillus larvae* White	*Bacillus alvei* Ches. & Chey.	*Bacillus para-alvei* Burnside	Filterable virus (*Morator aetatulae* Holmes)
Age of larvae at time of death	Usually die after cell is capped	Usually die while coiled in the cell, before cell is capped	Mostly unsealed but more in sealed cells than with European foulbrood	Usually die after capping of cell
Appearance of brood combs	Cappings become sunken and perforated. Dead brood in capped or perforated cells, or in cells uncapped by bees	Brood becomes spotted; many open cells with yellowish to dull-gray larvae. Few cell cappings may be perforated	Resembles combs with European foulbrood, although more sealed cells affected	Slightly irregular, ordinarily only few cells affected. Dead mostly in perforated or uncapped cells
Position of infected form in cell	Sticks to lower side and bottom of cell, stretched lengthwise in cell	Various positions; may be on side or bottom near opening of cell	Usually irregular, as in European foulbrood, or may be fully extended	Stretched lengthwise of cell, head prominently raised
Color of infected forms	Light brown to coffee brown; finally become dark brown to almost black	Yellowish white; finally change to brown or black	Reddish-brown to dark brown. Scales in unsealed cells lighter in color	Grayish to straw yellow, becoming grayish-black to black; head end usually black
Odor	Typical gluepot odor, especially in ropy stage	Sour to that of decayed meat; not always in evidence	Slight in unsealed cells, but very putrid in sealed cells	Slightly sour or none

Cuticle	Becomes soft and loses form	Remains entire, but becomes translucent with tracheae showing through	Becomes soft, and may be translucent	Remains entire and tough while contents are watery. Does not adhere to cell
Consistency	Sticky, ropy; stringing out 2 to 4 inches in viscid stage	Most unsealed larvae watery or pasty, seldom sticky; occasional sealed larvae may rope slightly	Dead larvae often become soft and watery. Sealed dead may be ropy	Watery to granular, never ropy
Pupae	Sometimes affected so that the tongue sticks up across the opening of the cell, a sure sign of the disease	Rarely affected	An occasional pupa is killed, but not so many as in American foulbrood	Seldom affected
Characteristics of the scales	Dark brown in color. Adhere tightly to cell wall; cannot be removed easily by the bees. Brittle	Segmentation and tracheae often visible. Dark brown to black, easily removed on drying. Tough and rubbery	Easily removed from the cells. Segmentation and tracheae sometimes visible	Tough, brittle, easily removed. Head end remains prominently tilted upward
Sex of larvae attacked	Usually only worker brood; rarely drone and queen larvae	All sexes	Generally worker and drone	Mostly worker; occasionally drone brood

* Most of the information in this table has been taken from a similar table by Eckert (1947*b*).
† Discussed in Chap. 11.

The organism has also been referred to as "Ishiwata's sudden death bacillus," and as "*Bacillus sotto*." Aoki and Chigasaki (1915), and others found the bacillus to be pathogenic for silkworms when the latter were experimentally infected. Other investigators experimentally infected the European corn borer but did not find the organism to be very pathogenic for this insect. The silkworm appears to be the most susceptible insect of those tested, but even here peroral infection is not easy. The injection of a drop of a suspension of the bacillus into the body cavity kills the larva in a few hours; in 3 or 4 hours at elevated temperatures. The effects of the bacillus appear to be due to a toxin of some kind, since injuries occur before the bacteria multiply in the general cavity. The body of the insect becomes blackened shortly before death.

Bacillus sotto grows on ordinary bacteriological media. Its exact identity, however, has never been determined, but it is probably not a species distinct from others that have been described. A strain of what appears to be the same species has also been observed in outbreaks of disease among silkworms in France. Also in France, Paillot (1942) isolated from a silkworm pupa a sporeforming bacterium which he named *Bacillus bombycoides*. It is similar in many respects to *B. sotto* and produces a toxin that causes lesions in the midgut epithelium.

While studying flacherie in silkworms in South China, Hartman (1931) isolated and described a bacillus which he named *Bacillus bombysepticus*. This bacterium was found capable of causing death of silkworms within 3 hours after the insects were fed large numbers of the bacteria. It is a gram-positive sporeforming rod having cultural and physiological characteristics typical of most species of *Bacillus*.

Bacillus ellenbachensis Gotth., probably synonymous with *Bacillus cereus* Fr. & Fr., has been reported as pathogenic for the silkworm, experimentally at least. Similar infections have been obtained by injecting into silkworms such common bacteria as *Bacillus megatherium* De Bary and *Bacillus mycoides* Flügge; and *Bacillus laterosporus* Laub. (= *B. orpheus* White) is pathogenic for this insect by both feeding and injection.

The Milky Diseases

Under the heading of "milky diseases" have been grouped a number of infections of scarabaeid grubs caused by certain sporeforming bacteria of the genus *Bacillus*. The best known of these are the milky diseases of the larva of the Japanese beetle, *Popillia japonica* Newm. These diseases constitute one of the most prominent means for the biological control of Japanese-beetle grubs in the northeastern part of the United States, where this insect is a serious pest of lawns, pastures, shrubbery, and other plants. The beetle was introduced into the United States from

Japan in 1916. It was first observed in a limited area in Burlington County, New Jersey, and has since spread over a large part of the New England states and into Canada.

The term "milky disease" is derived from the milky-white appearance assumed by the infected grubs. This opaque, chalky whiteness is the result of the accumulation of large numbers of the bacterial spores in the body cavity of the diseased larvae.

Early History. As early as 1921, it was known that the larva of the Japanese beetle was susceptible to certain diseases, supposedly caused by microorganisms of some kind (Smith and Hadley, 1926). The first studies on these diseases were undertaken by G. E. Spencer, at the Japanese Beetle Laboratory of the U.S.D.A. Bureau of Entomology in 1926 and 1927. He was able to isolate several species of bacteria from the affected insects. By inoculating healthy grubs with pure cultures, he found certain of the bacteria to be highly pathogenic. Spencer also observed the larvae to be attacked by fungi. In 1928, studies on the diseases of the insect were continued at the Japanese Beetle Laboratory by Henry Fox and R. W. Glaser in cooperation with the New Jersey Department of Agriculture. Although these men began working with bacterial cultures from diseased larvae, their attention was soon turned to a nematode (*Neoaplectana glaseri* Steiner) that was found parasitizing the insect.

In 1933, the work at the Japanese Beetle Laboratory was augmented by the cooperation of G. F. White, who, with I. M. Hawley (Hawley and White, 1935), divided the various infections encountered into three groups: the black group and the white group, both caused by bacteria, and the fungous group. The black group consisted of larvae that turned black in color during the course of the disease or soon after death. At least three different species of bacteria appeared to be responsible for these deaths. These bacteria were easily grown on ordinary culture media. The white group consisted of larvae that had an unnatural milky-white appearance. These were frequently found alive in the field. The body cavities of the insects were found to contain large numbers of bacteria that did not respond to any attempts to cultivate them. Here, it should be noted, is the first significant record of the group of diseases we are discussing. The fungous group contained larvae which bore tufts of fungous growth along their sides, and which, when dead, were firm, brittle, and thoroughly invaded with mycelial growth. Hawley and White also undertook field studies to determine the seasonal incidence of the diseases and the extent of the diseases in certain areas, and to explain the high mortality among larvae in certain field plots.

Although Hawley and White reported the black group to be the most prevalent, Hadley, in 1938, found the white group to be the most abundant,

especially in areas of longest Japanese-beetle infestations. Hadley concluded that the diseases in the white group were due to two, or possibly three, similar but distinct organisms. In 1940 Ralph T. White and S. R. Dutky showed the white group to consist principally of two types of infection. These have been designated as type A and type B milky disease. The causative agents of these two types were described by Dutky, who named them *Bacillus popilliae* and *Bacillus lentimorbus*, respectively. A third milky disease, caused by an unnamed bacillus, has been reported in *Odontria* grubs from New Zealand.

As soon as the true nature and cause of the milky diseases were determined, the Bureau of Entomology, in cooperation with certain state agencies, proceeded to develop methods for the mass propagation of the bacteria, particularly *Bacillus popilliae*, with the intention of using these organisms in the biological control of Japanese-beetle larvae. Methods of distribution were also worked out, and the effectiveness of the bacteria as a control agent was studied in the field. During this same period, other workers (*e.g.*, Beard, 1945) were investigating various aspects of the biological relations between the bacteria and their insect hosts. The reports on all this work form the basis of the account that follows.

Type A Milky Disease

As has just been explained, type A milky disease of the Japanese beetle is caused by *Bacillus popilliae* Dutky. It is the best known and economically most important of the so-called "milky diseases." The essential differences between it and the type B disease will be described in our discussion of the latter affliction.

Although the Japanese beetle appears to be the principal host of *B. popilliae*, other scarabaeid larvae are known to be susceptible to the bacillus. According to Dutky (1941), *Anomala orientalis* Wtrh., *Autoserica castanea* Arr., *Cyclocephala borealis* Arr., and *Strigodermella pygmaea* (Fabr.) have been found naturally infected with *B. popilliae*. Experimental infections have been tried successfully in the first three of these species, in *Odontria zealandica* White; in *Strigoderma arboricola* (Fabr.); and in several species of *Phyllophaga*, including *P. bipartita* Horn, *P. ephilida* (Say), *P. anxia* LeC., *P. fusca* Frohl., and *P. rugosa* Melsh. On the other hand, *Cotinis nitida* (Linn.) and *Macrodactylus subspinosus* are apparently not susceptible.

A bacillus similar to, or a variety of, *B. popilliae*, usually referred to as "atypical type A" or "type A (*Cyclocephala* strain)," has been reported by White (1947) as infecting larvae of *Cyclocephala borealis* Olive and *C. immaculata* Arrow in the field in eastern United States. White suggested

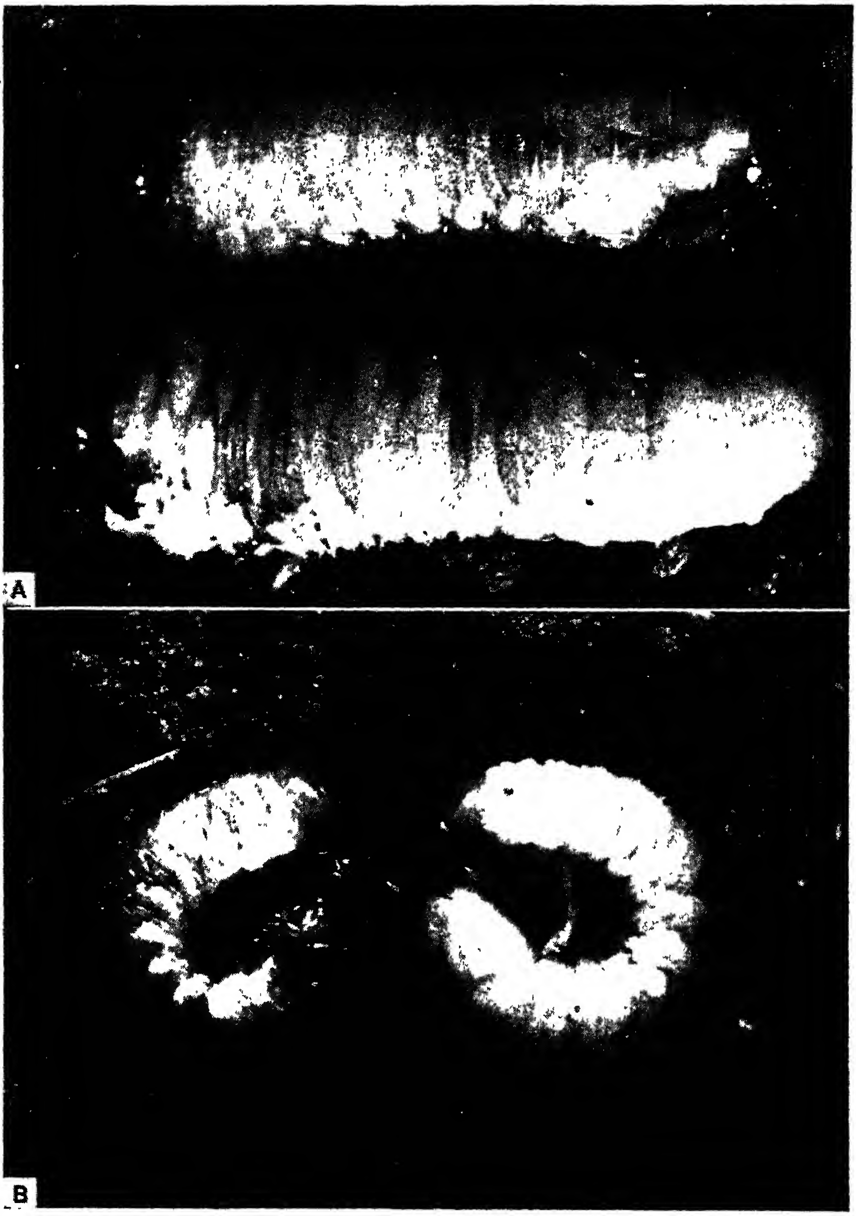


Fig. 76. Milky disease of Japanese-beetle grubs. *A*. Dorsal view of a healthy (top) and a diseased grub. Note that in the healthy larva the mid-line of the dorsum appears dark because of the transparent condition of the body fluid, which, in diseased larvae, is milky-white in appearance. *B*. Lateral view of a healthy (left) and a milky-diseased grub. (*From Wheeler and Adams, 1945; courtesy of E. H. Wheeler, New York State College Agricultural Experiment Station.*)

that the disease may be playing an important role in checking sporadic infestations of the larvae in many places.

Fig. 77. Lateral view of a healthy Japanese-beetle grub (left), and a diseased grub (right). Note the greater opacity of the diseased grub, particularly in the legs. (*From Beard, 1945.*)

Symptoms. Judged from external appearances alone, very little difference may be visible between a healthy Japanese-beetle grub and one infected with *B. popilliae*. With a little experience, however, a few signs or symptoms may be noted that distinguish the two. Upon examination of the pericardial region and the posterior segments of a diseased grub, it will be noticed that the increased turbidity of the blood tends to obscure the dorsal vessel and the rectal sac, readily visible in the healthy insect. The opacity of the legs is also increased. As the disease progresses the larva assumes a milky-white appearance which may be distinguished from fat accumulations by the proper manipulation of the specimen. If the posterior segments are gently constricted between the fingers, the fat tissue may be seen to move as a unit, whereas the turbid blood flows irregularly in the spaces of the body cavity. The turbidity of the blood increases progressively until the larva is almost uniformly opaque and the insect becomes moribund.

Fig. 78. Japanese-beetle grub in an advanced stage of type A milky disease, showing uniform opacity over the entire body. (*From Beard, 1945.*)

Not until within a few days of death is the activity of the larva appreciably affected. About this time it becomes sluggish, ceasing its spontaneous movements and then losing its response to tactile stimulation. Its color becomes slightly brownish, except in the lower parts of the body, which remain chalky-white as a result of the settling out of spores in the almost static blood.

If one pulls off the leg of a diseased grub, the drop of body fluid that oozes from the tip of the dismembered leg has an opaque white appearance.

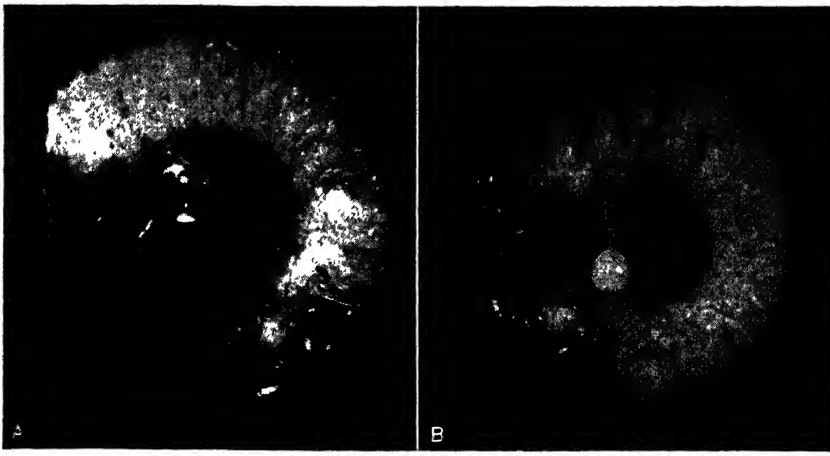


Fig. 79. Healthy (*A*) and milky-diseased (*B*) Japanese-beetle grubs. A drop of body fluid from each grub is shown oozing from the tip of a cut leg. Note the opaque, cloudy aspect of the fluid from the diseased insect. (*From Wheeler and Adams, 1945; courtesy of E. H. Wheeler, New York State College Agricultural Experiment Station.*)

A similar drop from the cut leg of a healthy larva is water-clear or only slightly cloudy. When the blood from a diseased insect is examined under a microscope, it is found to be filled with slender nonmotile rods, and highly refractile, spindle-shaped spores. It is these spores which impart to the blood of the diseased grub its characteristic milky-white appearance. A further distinguishing difference between the blood of healthy grubs and that of diseased grubs is seen if the drops of blood are exposed to the air. A drop of blood from a healthy grub soon becomes very dark in color after such exposure. On the other hand, blood from a grub with milky disease usually fails to undergo this change (Fig. 80).

Although the adult beetle is known to be susceptible to *B. popilliae*, symptoms in this stage of the insect are not very discernible or distinctive. It is known that the diseased beetles have a much shorter life than do noninfected beetles.

The Causative Agent. The causal relationship between the disease

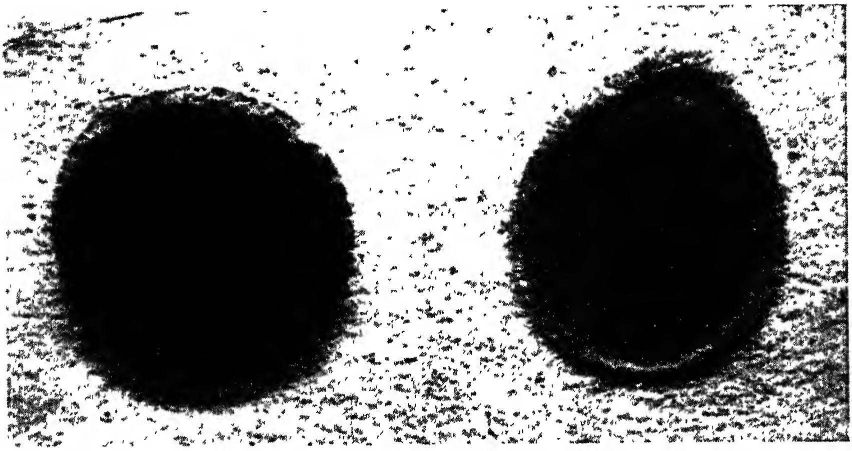
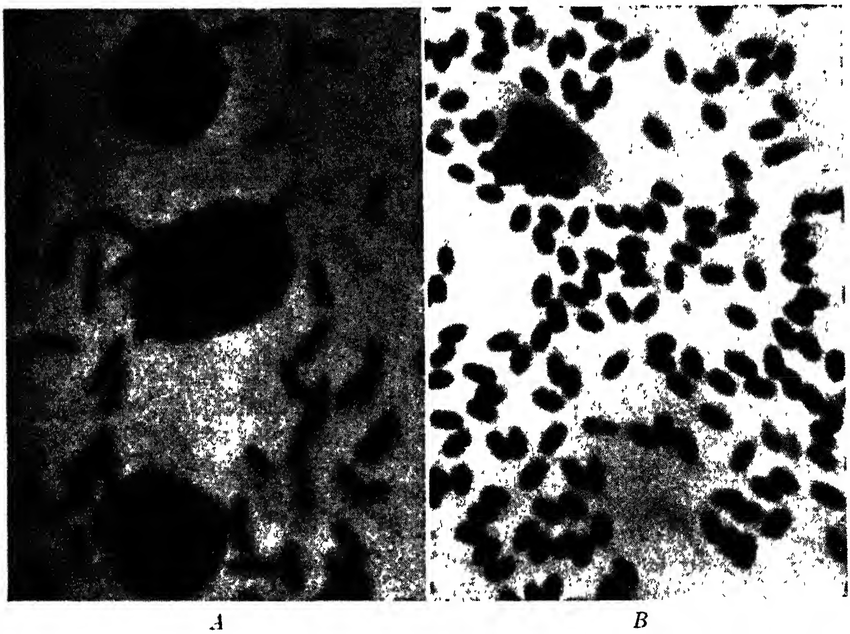


Fig. 80. Drops of blood (exposed to the air) from Japanese-beetle larvae. Blood from a healthy larva is shown on the left; that from a diseased larva, on the right. (*From Beard, 1945.*)



A B

Fig. 81. *Bacillus popilliae* Dutky, the causative agent of type A milky disease of the Japanese beetle. A. Part of a blood smear from an infected larva showing the vegetative rods of *B. popilliae*. (The three large structures are blood cells.) *B*. Spores of the bacillus stained with carbon fuchsin. (*From Beard, 1945.*)

and the sporeforming bacterium found in the larval blood was demonstrated by Dutky (1940), who named the organism *Bacillus popilliae* after the generic name of its host, *Popillia japonica* Newm. Dutky described the bacterium as follows:

The vegetative form of the organism is a slender, nonmotile rod occurring singly or in pairs. In the living condition the rods measure 0.9 by 5.2 microns. When fixed by Schaudinn's solution and stained by Hucker's crystal violet, the dimensions are about 0.3 by 3.5 microns. The mode of division appears to be by plate formation rather than constriction, and is evidenced by the squareness of adjoining ends of the paired cells. After separation the ends are somewhat rounded. The cytoplasm in young cells is homogeneous and stains uniformly with Gram stain; in older cells granules are often found, and after fixing and staining, unstained areas are seen which divide the cell into two unequal sections.

The rods become swollen at sporulation. When the cell begins to swell, the spore becomes visible as a slightly refractile vacuole equal in size to the mature spore. As sporulation proceeds, the vacuole becomes more and more refractile until a definite spore is observed. At this time the cell has a pronounced spindle shape, and the spore is located somewhat terminally. One end of the cell broadens, and the cell becomes more pyriform than spindle-shaped. A granule is now observed in the broadened end, which grows until it is about half the size of the spore. With the development of the granule the spore assumes a more nearly central position. The cytoplasm about the spore becomes increasingly refringent.

After the completion of the refractile body and the increase in density of the cytoplasm surrounding the spore, no further morphological changes occur. In the fresh state the spore and granule are homogeneous in internal structure, and they do not take up either stains or iodine. The spore is surrounded by a halo formed by the encircling protoplasm, but it is very definite in outline. Spores free from the sporangium have never been observed. The size of the unstained sporangium is 1.6 by 5.5 microns, and that of the endospore 0.9 by 1.8 microns. When fixed by Schaudinn's solution and stained with Hucker's crystal violet, the refractile body and spore remain unstained, but the latter is obscured by the deeply stained surrounding protoplasmic layer. When fixed and stained, the spore-bearing cells are approximately 1.3 by 3.6 microns in size. When stained by Dorner spore stain, both the refractile body and the spore retain the stain, whereas the cytoplasm is completely decolorized. The membrane of the vegetative rods and both the membrane and the refractile body of the spore-bearing forms are resistant to the action of alkalies, remaining intact for at least 2 days in 10-percent sodium hydroxide solution.

The spores are heat-resistant, withstanding temperatures of 80°C. for 10 minutes, as shown by the production of the disease in larvae by inoculation of heated spore suspensions. The thermal death point of the spores has not been determined. The spores are also resistant to desiccation. Spores in blood films dried for periods as long as 42 months have given consistently high infection when moistened and inoculated into healthy larvae.

Beard (1945) has amended Dutky's description by explaining that

The entire spore-structure is pyriform to spindle-shape, consisting of a sporangium containing an endospore and a refractile body. When stained by a carbol fuchsin spore stain, the endospore alone remains prominent. In unstained preparations, the external protoplasm is only faintly visible, and the prominent endospore and refractile body are so placed as to suggest a footprint in outline. Because of this

Fig. 82. Nigrosin preparation of the spores of *Bacillus popilliae* Dutky. Toward the left end of the figure may be seen a spore with the footprint appearance characteristically seen in unstained smears. (*From Beard, 1945.*)

characteristic shape, the spores of *B. popilliae* may be distinguished from other bacteria or debris with reasonable certainty.

Bacillus popilliae has been successfully cultivated on media held under anaerobic conditions or containing substances, such as unheated egg yolk, which create a reduced oxygen tension in the medium.[1] Beef-infusion agar, with or without dextrose and/or peptone, serves as a satisfactory basal medium. On such media the organism forms small discrete colonies of nonmotile slender rods only. As yet, no one has succeeded in producing spores on artificial media.

Pathogenesis of the Disease. In nature, infection of a Japanese-beetle grub with *Bacillus popilliae* ordinarily follows the insect's ingestion of the bacterial spores as it feeds on the roots of grass or other plant material. Thus the route of infection may be said to be by way of the alimentary

[1] Because of its anaerobic or semianaerobic requirements, *Bacillus popilliae* may possibly be more closely related to members of the genus *Clostridium* than to those of the genus *Bacillus*.

tract. Contrary to what was first believed to be the case, larvae apparently do not become infected by the bacteria in the soil entering their bodies through wounds in the integument although, of course, infection can be induced artificially by hypodermic injection into the body cavity. On the other hand, healthy grubs that bite diseased individuals may, by getting a mouthful of infectious blood, become infected in this manner.

The ingested bacteria penetrate the gut wall and enter the hemocoele in their vegetative form. Spores that are ingested apparently first germinate in the lumen of the gut or its diverticula, and reach the blood in their vegetative form. Penetration of the gut wall is believed to take place through the Malpighian tubules or at least somewhere in the posterior

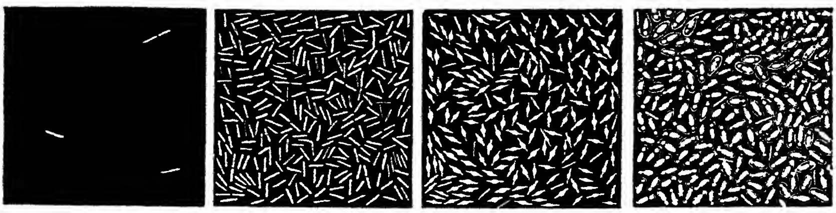


Fig. 83. A diagrammatic representation of the developmental phases of *Bacillus popilliae* Dutky. (*From Beard, 1945.*)

region of the ventriculus or the anterior region of the hindgut. At any rate, it is quite certain that the bacteria do not germinate in and penetrate the rectal sac and rectum (Beard, 1945).

After the vegetative bacteria have gained entrance into the body cavity, they multiply rapidly in the blood, producing a bacteremia or septicemia. When the vegetative forms have become exceedingly numerous, the bacteria sporulate. This sporulation occurs as a wave and continues until the definitive spore stage is reached by all those bacteria undergoing sporulation. It is at this time in the disease that the blood of the insect assumes its milky-white appearance and has a characteristic granular aspect when viewed under the low power of the microscope. The form characterizing the completion of this developmental cycle is characterized by the "footprint" endospore and refractile body surrounded by a sporangial membrane. It is doubtful whether the completed spores, once formed, are capable of germinating in the diseased grub and of repeating the cycle, since, as pointed out by Beard, intermediate sporulating forms are not in evidence after sporulation is once complete.

The relationship between time and the development of the disease varies in nature and is dependent largely upon temperature. Dutky (1940) observed that when healthy larvae are inoculated with spores of

B. popilliae and held at 30°C., no change is apparent in either the morphology or the number of spores for about 12 hours afterward. There is then a gradual decrease in the number of spores until, after 30 hours, about half the orginal number remain, and vegetative rods are seen in small numbers, usually in pairs. After 48 hours, about one-third of the original number of spores remain and the rods are present in large numbers. On the third day the rods begin to swell, and 24 hours later sporulation occurs and continues until the number of spores reaches a maximum about 13 to 16 days after inoculation. The turbidity of the blood is usually apparent on about the sixth day, with the opacity increasing until sporulation is complete, at which time the total number of spores in the blood has been calculated to range from 500 million to 20 billion, averaging from about 2 to 5 billion, per larva. A few rods ("shadow cells") apparently incapable of sporulation remain among the vast number of spores. Macroscopic symptoms of the disease, occurring on the sixth day at 30°C., show up in 4 days at 34°C., in 9 days at 25°, in 11 days at 22°, and in 14 days at 17°, the temperature range for the development of the disease being, for all practical purposes, from 16 to 36°C. Dutky interpreted his data as indicating a linear relationship between the time of the development of the disease and the temperature. Beard (1945), however, on the basis of periodic microscopic examinations, found that at any given stage of the disease development, the time required seemed to follow more of an exponential function of the temperature.

The pathogenic effect of *B. popilliae* on its grub host appears to be of the nature of the general suppression of the functions vital to the insect, rather than being due to the action of exotoxins. Unlike larvae suffering from typical bacterial septicemias, grubs infected with the milky-disease organism do not die at the height of the infection but may live for a shorter or longer time (for weeks or months at lower temperatures) in the diseased condition. Grubs infected during the earlier instars usually die more quickly than do older ones. Beard has presented data showing that grubs do not die at any definite time following the development of the disease but live varying lengths of time, presumably depending upon the vigor of the individuals. They usually continue to feed until they become moribund. Some infected grubs may transform to pupae and adults, but metamorphosis may be inhibited and prevented. This depends on how far along the disease is. Larvae containing mature spores are nearly always unable to pupate. Molting of the younger larvae is similarly inhibited.

Factors relating to the general susceptibility and resistance of Japanese-beetle grubs to infection by *B. popilliae* have had only limited experimental consideration. Beard (1944, 1945) has studied the effect of the spore dose

on the incidence of the disease, and he observed that the probability of a grub's becoming infected increases with the spore dose, whether this is received by injection into the body cavity or by ingestion into the gut along with food. This holds true for all three of the larval instars, the susceptibilities of which are of the same order of magnitude when reared under equivalent conditions. The effect of the preliminary feeding of grubs on their susceptibility to *B. popilliae* appears to be insignificant. Grubs removed from cold storage at the time of inoculation exhibited about the same degree of susceptibility as did those not subjected to storage. Of course, susceptibility must always be considered in terms relative to the virulence of the bacterium. In this connection it is of interest to note that Beard found the virulence of *B. popilliae* for the Japanese beetle to be less than that of *B. larvae* for the honeybee. Whereas only about 25 spores of the latter bacillus are required to produce foulbrood in 50 per cent of the bee larvae, 11,000 *B. popilliae* spores are required to cause milky disease in the beetle grubs when injected parenterally. No clear demonstration of ordinary immunity principles has been made in the case of type A milky disease in the Japanese beetle. Beard noted some evidence of resistance to the disease on the part of the beetle grub, but this probably does not represent an immunological phenomenon in the usual sense. As far as is known, once a grub becomes infected with *B. popilliae*, it does not overcome the disease.

Pathology of the Disease. Specific pathological and physiological effects of the disease on the host grubs apparently are few and mostly of a negative character. There is need for a closer analysis of the histopathological changes that probably take place in certain of the insect's vital tissues.

No gross necrosis or degenerative changes have been observed in any organ or tissue of the diseased insect. Even the post-mortem changes are few, and putrefaction sets in only as the adventitious bacteria of the gut break through the gut wall and flourish in the remains of the insect.

Beard (1945) concluded that changes in the inorganic chemical constituents of the blood, the number of blood cells, the osmotic pressure of the blood, blood pH, or manner and time of blood coagulation are too slight to account for the over-all effect of the disease. He did find that the disease commonly disturbs at least one oxidizing enzyme system; and, since oxidizing enzymes are probably necessary for molting, metamorphosis, and the full realization of life expectancy, Beard believes that the effects of the disease may be caused by the destruction of one or more enzyme systems.

Properties of Spores. In order to understand properly the manner in which *Bacillus popilliae* is transmitted, and the methods of using the bacillus against the beetle grubs, the student should keep in mind some

additional general characteristics and properties of the spore. This resistant structure of *B. popilliae* is the stage that makes it possible for the bacillus to maintain itself effectively in nature.

If the spore-containing blood of a grub is drawn out as a film on ordinary glass slides and allowed to dry, the spores will retain their viability and potency for many months. Actual tests have shown the spores to be still capable of causing disease after being held thus for 58 months. Similarly, when incorporated in soil and exposed to the weather, the spores are able to retain their potency for extended periods of time. It was noted by Beard (1945), however, that fresh spores were six times as potent as were spores that had been incorporated in a dust for 2 years. Also spores exposed to ultraviolet light suffer reduction of potency, as they do when heated at temperatures above 90°C. There is also some loss of potency when the spores are kept refrigerated in a water suspension. Although a low pH seems to affect the potency of the spores adversely, from a practical point of view the pH of most soils is within the range at which any harmful effects are not likely to occur, and hence this factor can largely be disregarded. The effect of successive passages of the bacteria through a series of hosts on the potency of the spores is not clear. Increased potency has been observed, but this has not been maintained consistently.

Fig. 84. Stained smear of *Bacillus popilliae* Dutky in the blood of a Japanese-beetle larva, showing both rod and spore forms. (*From Dutky, 1940; courtesy of C. H. Hadley, U.S. Department of Agriculture.*)

As has already been mentioned, the average number of spores that develop within a single grub is in the neighborhood of 2 to 5 billion. The number of spores produced does not seem to be correlated with the body weight of the host, the temperature of proper incubation, or the size of the inoculum.

Transmission and Natural Dispersion of the Bacillus. In nature, the principal source of infection for type A milky disease is probably soil contaminated by the disintegrating bodies of diseased grubs containing mature spores. The spores thus liberated become incorporated in the soil and may then eventually be ingested by a susceptible grub. The rate

of this type of transmission is probably affected by the rate of the insect's decomposition, the moisture conditions, and the microbial activity normally present in the soil. Although the droppings of living diseased grubs are not known to contain the bacillus, such larvae may nevertheless serve as a source of infection when they are bitten by healthy grubs which thereby ingest the bacteria-containing blood. Both the vegetative and spore forms of the bacillus may be transmitted in this manner. Beard (1945) has shown that third-instar grubs containing only vegetative rods transmit the smallest amount of disease. Intact grubs, whether dead or alive, containing mature spores, were responsible for an intermediate amount of disease. Spores from disintegrated larvae and in direct contact with the soil gave the highest incidence of disease.

Of considerable importance in the transmission and rapid spread of the disease is the grub population and the inoculum potential. The more concentrated the population of susceptible individuals, the more rapid is the spread of the disease. Beard found this to be true when he also determined that a high inoculum potential also favors its spread. He noticed, however, that a heavy population can compensate for a low inoculum potential and that, conversely, a heavy inoculum potential can compensate for a low population in causing a resultant high incidence of milky disease. In some of his experiments Beard observed that an increasing inoculum did not result in a progressive increase in the incidence of disease. Instead, a period of increasing morbidity was followed by a decline. This may be explained by the fact that at first the infection rate exceeds the mortality rate; then the mortality rate exceeds the infection rate. This last event may, in part, be due to an accumulation of the more resistant grubs.

Transmission of *B. popilliae* from the larval to the adult stage is known to take place in light infections or in infections initiated late in larval life. That the adult beetle is a factor in the natural dispersion of the bacillus was shown by Langford, Vincent, and Cory (1942) when they discovered the disease in field-collected adults and the fact that larvae held in soil mixed with spores from diseased adults develop the disease. As these authors point out, this is supported by the close relationship existing between the migratory habits of the beetle and the incidence of the disease in the peak infestations within the area of continuous distribution.

The dispersion of *B. popilliae* may also take place by the movement of topsoil (the spores usually tend to remain more concentrated in the top 2 inches of soil) by wind, water, or man. White and Dutky (1940) cite field and laboratory observations which prove that birds and insects may aid the dispersion of the bacillus. Viable spores were voided in the droppings of chickens and starlings that had been fed milky-diseased

larvae. That ants may be important in the local spread of the bacteria is evidenced by the fact that they have been seen dragging dead diseased grubs for distances of at least 10 feet. Skunks, moles, and mice are known to feed upon the larvae and hence probably play some role in the dispersion of the organisms.

Use of *Bacillus popilliae* in Control of Beetle. A considerable amount of time and money has been expended in research on the milky diseases of the Japanese beetle because of the possibility that herein lay a promising means of controlling this very destructive insect. The use of chemical insecticides, trapping methods, insect parasites (*Tiphia*), and nematodes had not proved adequate to cope with the seriousness of the situation. The hope that *Bacillus popilliae* would be an effective adjunct to the efficacy of these other agents has been realized, although it has not replaced them. In fact, the most effective control appears to be a combination of the use of chemicals for rapid control and the use of the milky disease for permanent or continuing control over long periods of time. Of course, an additional incentive for the development of effective bacterial control has been the relative inexpensiveness of this method as compared with most other methods.

The central agency in the milky-disease fight against the Japanese beetle has been the Bureau of Entomology and Plant Quarantine of the U.S.D.A. At their Moorestown, New Jersey, laboratories, this group of workers (G. F. White, Hawley, Dutky, R. T. White, Hadley, Dobbins, and McCabe) directed their work toward the evolution of methods and procedures by which the bacteria could be used effectively in the field. The U.S.D.A. cooperated with several other Federal and state agencies in a program of distributing the spores of the bacillus over a large part of the area infested by the beetle.

Since no one has yet succeeded in causing *B. popilliae* to produce spores when grown on artificial media, the first requirement to be met was that of finding ways to produce spores in large quantities. Following this, methods had to be devised for preparing the spores in suitable form for storage and field distribution. This was accomplished by 1939, and the essential features of the method were patented. By 1944 effective spore dusts were obtainable for commercial sources.

The methods used consist essentially of the following major steps (according to Dutky, 1942, and others): Stock cultures of the bacillus, kept as films of dried larval blood on glass slides, are suspended in water and used to inoculate healthy Japanese-beetle grubs. A special "micro-injector," furnished by the Federal Bureau, is used for the inoculating. The grub to be inoculated is forced onto the needle point of the loaded syringe so that the needle enters through the dorsal portion of the suture

between the second and third posterior abdominal segments. A dosage of about 0.03 milliliter of spore suspension (approximately 1 million spores) is then introduced into the body cavity of the insect. The inoculated larvae are incubated in boxes which are separated into soil-filled compartments by means of cross-section separators and which have a capacity of 500 grubs. Incubation is at 86°F. for from 10 to 12 days. After incubation, the boxes are broken down, and the diseased grubs are screened out of the soil and dropped into a battery jar of ice water, which inactivates them. In the jars the larvae are packed in ice and held in a refrigerator at temperatures of approximately 32 to 35°F. until used. When sufficient numbers of diseased grubs have been accumulated, they are crushed by running them through a meat chopper, and then they are suspended in water. After the suspension is standardized, it is added to the carrier (calcium carbonate) so that the mixture will contain a billion spores per gram of dry material. The dried dust concentrate is mixed with talcum powder, or other suitable dry carrier, and is stored until used. This final mixture, as prepared by the Bureau, contains approximately 100 million spores per gram.

The spore dust is usually applied with a hand corn planter of the rotary type so adjusted as to deliver 2 grams of material per spot. It is applied at intervals of 10 feet, which enables the bacillus to disseminate throughout the treated area in three seasons (White and McCabe, 1943). The spore dust may also be mixed with fertilizer and the two materials applied together (White, 1948). The manner of treatment and the size of the areas treated are varied according to the specific requirements of the situation involved and the type of program required by the different states.

Ever since the program of distributing the spore-dust mixture was begun by the Federal government in 1939, reports on its success have been made regularly. Each year the area has been increased until by 1948 more than 90,000 sites covering almost 74,000 acres have been treated in 12 states and the District of Columbia. Additional acres have been treated with spore dust produced and distributed by commercial concerns. Furthermore, it should be realized that the disease is extending itself naturally and that a great deal of natural control is taking place as the result of the bacterium's activity, without the aid of man.

A specific example of the type of encouraging result obtained is that recorded for one of the park areas in the District of Columbia. Before spore-dust treatments in 1940, the Japanese-beetle grub population was as high as 44 larvae per square foot. By 1943 the number of grubs had dropped to about 5 per square foot. In general, according to U.S.D.A. officials, the milky-disease distribution program is giving relief to fruit growers, farmers, and homeowners at a much earlier date than would be

the case if the disease were left to spread by natural means. Furthermore it is preventing Japanese-beetle infestations from reaching as high levels as would be the case without the disease, and the infestations are being reduced to negligible proportions much more promptly. The final appraisal of the ultimate benefits of this method of biologically controlling the beetle has yet to be made. Further aspects of the use of *B. popilliae* in the biological control of the Japanese beetle will be discussed in Chap. 14.

Type B Milky Disease

Type B milky disease of the Japanese beetle is caused by *Bacillus lentimorbus* Dutky, a sporeforming bacterium first described and named by Dutky in 1940. The great majority of the research on the milky diseases has been done on the type A disease caused by *Bacillus popilliae,* with the almost complete abandonment of any consideration of the type B disease as a control agent. Consequently, for most of the information concerning *B. lentimorbus* and the disease it causes, we are limited to Dutky's (1940) original account.

Symptoms. From their gross appearance Japanese-beetle grubs suffering from type B milky disease, when observed in the late summer and fall, cannot be distinguished from those having the type A disease. Diseased larvae that have overwintered, however, have a distinctly different general appearance. These larvae are characterized by a muddy-brown coloration instead of one that is milky white. Diseased grubs collected in the very early spring are usually of a milky-white color. If these insects are held at room temperature they darken rapidly, and by the end of 2 or 3 weeks they have the chocolate-brown color characteristic of type B–diseased larvae during April and May in northeastern United States. These dark-brown larvae may be alive and active; eventually, however, the insect is unable to pupate and dies.

If infectious material from one of these brown grubs is inoculated into the body cavity of healthy larvae, they first develop the milky-white condition. With an increase in temperature, they too become chocolate brown in color. The brown condition, however, is not directly reproducible in the newly inoculated host.

According to Dutky, the darkening of the diseased grubs is caused by the extensive formation of blood clots that are brown to jet black in color. The accumulation of these clots in the insect's appendages blocks the blood circulation, and the gangrenous condition that results causes the affected parts to blacken.

The Causative Agent. *Bacillus lentimorbus* Dutky is a sporeforming rod but, unlike *B. popilliae* Dutky, it does not have the refractile body so prominent in the latter. The sporangium is decidedly more spindle-

shaped than is that of the type A organism. When stained with crystal violet the sporebearing rods color strongly and evenly and have a distinct lemon shape. The size of the vegetative rod is approximately 1.0 by 5.0 microns. That of the endospore is 0.9 by 1.8 microns, and of the sporangium 1.4 by 3.9 microns. The bacillus is gram-positive. All attempts to cultivate this organism on artificial media have been unsuccessful.

The spores of *B. lentimorbus* are capable of withstanding a temperature of 85°C. for 10 minutes when heated in saline suspensions. They are known to resist desiccation for at least 42 months.

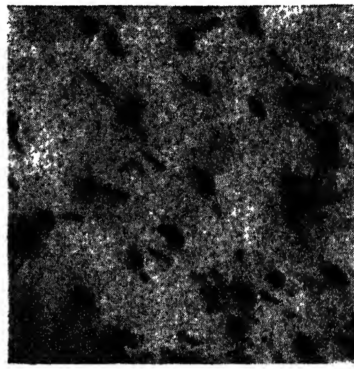


Fig. 85. Stained smear of *Bacillus lentimorbus* Dutky, the cause of type B milky disease, showing both vegetative rods and spores. (*From Dutky, 1940; courtesy of C. H. Hadley, U.S. Department of Agriculture.*)

Pathogenesis of the Disease. The exact route by which *B. lentimorbus* enters the body cavity of its host has not been determined. Since, in addition to direct inoculation, infection may be initiated by feeding the bacteria, it may be assumed that the digestive tract or some part of it probably serves as the main portal of entry. Once within the body cavity of the grub, the bacteria multiply in the blood in much the same way as does *B. popilliae*, except that the type B organism may also attack other tissues. As has already been mentioned, brown to black blood clots are formed and accumulate in the appendages of the insect, blocking the circulation and producing a gangrenous condition that eventually assists in bringing about the death of the host.

After inoculating larvae with a dose of 2 million spores and holding them at 30°C., Dutky made periodic examinations of the blood and observed the several stages of the organism's development in the host. After inoculation, a gradual reduction in the number of spores was observed for 2 days, with vegetative rods, mostly in pairs, appearing on the third day. Since the adjoining ends of the paired cells were truncate, division probably occurs by plate formation rather than by constriction. As time passes, the number of rods increases. On the fifth day the rods begin to swell, and vacuoles may be noted in a few cells. The number of sporulating rods increases, and on the ninth day they are present in sufficient numbers to give the first external symptoms of the disease. At 30°C., the total number of spores per larva usually does not exceed 1 or 2 billion, even after 2 weeks at this temperature. At temperatures somewhat below

30°C. the number of spores continues to increase after the visible symptoms first appear, until they reach numbers between 5 and 10 billion spores per grub.

If mature third-instar larvae are inoculated, the insect is frequently able to pupate before it succumbs to the disease.

Although the exact maximum and minimum temperatures for the development of *B. lentimorbus* have not been determined, Dutky (1940) found that they approximate a range of between 12 to 16°C. minimum and 30°C. maximum. Thus the range for the type B organism is smaller than that of the type A organism (16 to 36°C.).

New Zealand Milky Disease

Under the temporary heading of "the New Zealand milky disease" may be considered an infection occurring in the larva of *Odontria zealandica* White, and first described by Dumbleton in 1945. The disease is caused by a sporeforming bacterium that is similar to, but distinct from, *Bacillus popilliae* and has been found present in several localities in New Zealand. Although clearly distinct from any previously described insect pathogen, the New Zealand bacillus unfortunately has not been named. Dumbleton has described it as possessing a spherical paraspore (that of *B. popilliae* is hemispherical or subconical), and not being infectious for the Japanese beetle, *Popillia japonica* Newm.

The symptoms of the infection in *Odontria* are in many respects similar to those of the milky diseases of the Japanese beetle. The spores are apparently ingested by the larvae and, after germinating, penetrate to the body cavity where the vegetative rods multiply in the blood. Later, as the rods sporulate, the larvae take on the milky-white appearance characteristic of the disease. This white opacity is generally first noticeable in the dorsal thoracic region. The larvae may remain firm and active for some time in spite of the infection. In time, and especially at high temperatures, the tissues disintegrate and the body becomes flaccid in consistency and brownish in color. Death comes slowly and, as with the other milky diseases, usually retards the growth and suppresses the metabolism of the host.

The distribution of the disease as it occurs in New Zealand has been studied only in limited areas. In these areas Dumbleton (1945) found the infection to be naturally present in amounts up to 38 per cent of the grubs present. In some areas, however, the disease has very low incidence in spite of its wide distribution and high host population. In other areas it appears to be of more importance as a control factor. The artificial distribution of the bacterium on a wide scale has not been practiced.

Infections Caused by *Bacillus cereus* Fr. & Fr., *Bacillus subtilis* Cohn *emend.* Praz., and Related Bacilli

Bacillus cereus Fr. & Fr. is a widely distributed sporeforming organism and is the most common species of the genus *Bacillus* found in the soil. It is frequently confused with another common sporeformer, *Bacillus subtilis* Cohn *emend.* Praz., which has a similar habitat. Undoubtedly

Fig. 86. Vegetative form of *Bacillus subtilis* Cohn, which under certain natural and experimental conditions may be pathogenic for insects. The vegetative form of *Bacillus cereus* Fr. & Fr. is morphologically similar to *B. subtilis*.

many of the sporeforming bacilli isolated, described, and given different names by early students of insect pathology and insect microbiology are in reality one or the other of these two species. This probable synonymy is difficult to ascertain from the literature alone. It has, however, been accomplished with a few insect pathogens, and these instances with regard to *B. cereus* may be considered briefly here. It should be pointed out that infections of insects have been reported in which the bacterium involved was recognized to be *Bacillus cereus*. For example, Babers (1938) reported a septicemia in the southern armyworm, *Prodenia eridania* Cram. and in the American cockroach, *Periplaneta americana* (Linn.), caused by this bacillus. Similar infections have been noted in cultures of the Indian mealworm, *Plodia interpunctella* (Hbn.). In addition, *B. cereus* has been isolated from apparently normal healthy insects and

ticks (Steinhaus, 1946*b*). *Bacillus mycoides* Flügge, now considered a variety of *B. cereus*, has been found experimentally pathogenic for the silkworm and for wax-moth larvae, *Galleria mellonella* (Linn.). The last-named insect is also susceptible to injections of *Bacillus pumilus* Gotth. (*B. mesentericus* Chest.). Also possibly synonymous with *B. cereus* is *Bacillus hoplosternus* Paill., isolated by Paillot (1919) from diseased cockchafers, *Melolontha melolontha* (Linn.), and found to be pathogenic for several lepidopterous larvae (*Nygmia*, *Malacosoma*, *Arctia*, and *Vanessa*). The gypsy-moth caterpillar *Porthetria dispar* (Linn.) showed a definite immunity to the bacillus. *Bacillus ellenbachi* Saw., referred to by Sawamura (1906) as producing a flacherie in silkworms, may also have been *B. cereus*.

Bacillus subtilis has also been reported as being associated with a number of healthy insects and ticks and has been found pathogenic for such insects as larvae of the wax moth, *Galleria mellonella* (Linn.), and the mealworm, *Tenebrio molitor* Linn., when inoculated into these insects. Masera (1934*d*) found the mealworm to be susceptible to outbreaks of septicemia caused by *B. subtilis* when the insect was subjected to such maltreatment as excessive temperatures. Experimentally this insect may be infected by direct inoculation and somewhat less effectively by ingestion.

"Bacillus thuringiensis." In 1915(*b*) Berliner, in Germany, described a sporeforming bacterium, which he named *Bacillus thuringiensis*, from diseased larvae of the Mediterranean flour moth, *Ephestia kühniella* Zell. Infection took place following the ingestion of the bacillus or its spores. A culture of this organism obtained from Mattes was studied by Smith, Gordon, and Clark (1946), who found it and Berliner's description to conform to their conception of *Bacillus cereus*. Whether or not the strains associated with the flour-moth larvae have a greater pathogenicity for insects than do ordinary strains of *B. cereus* from the soil has not yet been made clear. The insect strains do not appear to lose their virulence to any great extent when grown continuously on artificial media. Since *Bacterium* [*Bacillus*] *ephestiae* M. & C., isolated from the same insect by Metalnikov and Chorine (1929*a*), has been found to be the same as *Bacillus thuringiensis* Berl. (Ellinger and Chorine, 1930), it may be assumed that this organism also is synonymous with *Bacillus cereus*. All these strains are large, motile, gram-positive, sporeforming rods which grow well on ordinary bacteriological culture media.

Under the name *Bacillus thuringiensis* (and *Bacterium thuringiensis*) the organism being considered was tried by a number of investigators as a means of controlling several insect pests. Shepherd, in 1924, mentions its use for the control of *Echocerus cornutus* (Fabr.) in Germany. Husz (1927), after demonstrating the marked susceptibility of the European

corn borer, *Pyrausta nubilalis* (Hbn.), suggested its use for combatting this insect. He subsequently (1929, 1930) reported favorable results in this regard by using spore dusts and sprays. Confirmatory results were obtained by Metalnikov and Chorine (1929*a,b*), who also found the bacillus pathogenic for larvae of *Porthetria dispar* (Linn.), *Aporia crataegi* Linn., and *Vanessa urticae* (Linn.). Since certain grasshoppers, mosquitoes, and beetles were not susceptible to the pathogenic action of the organism, these workers concluded that *B. thuringiensis* was virulent only for larvae of Lepidoptera.

***Bacillus* "C."** Another sporeforming bacterium now known to be a strain of *Bacillus cereus* is that studied by Sokoloff and Klotz (1941, 1942) and designated by them as *Bacillus* "C". These workers first isolated the organism from the soil, and later from the California citrus red scale, *Aonidiella aurantii* (Mask.), for which it was found to be pathogenic. The bacillus was reported to be capable of invading and destroying the adult scale insects on lemons under laboratory conditions. It was believed that the lethal effect of the bacterium was related to the reduction of nitrate within the body of the insect. Efforts to repeat the results obtained by Sokoloff and Klotz have been made (Steinhaus and Snyder, 1947), but the reported invasive properties of the bacillus could not be demonstrated. Similar lethal effects were obtained with broth cultures of the organism, but these were shown not to be associated with any real invasion of the insect by "*Bacillus* 'C'." In fact, the same lethal effects could be obtained with bacteria-free filtrates of the broth cultures.

Other Bacilli. In the course of attempts to find some satisfactory control of the European corn borer, *Pyrausta nubilalis* (Hbn.), several strains of sporeforming bacilli were isolated which showed varying degrees of pathogenicity for this insect. Although most of these bacteria were given names, it is probable that some of them are in reality varieties of strains of some of the more common and better known sporeformers. Some of these bacteria were originally given the generic name *Bacterium*, but the fact that they are aerobic sporeformers places them in the genus *Bacillus*. The species concerned include *Bacillus canadensis* (Chor.), *B. christiei* (Chor.), *B. italicum* (M., E., & S.), *B. ontarioni* (Chor.), and two different strains each of *B. cazaubon* (M., E., & S.) and *B. pyrenei* (M., E., & S.). The relative value of these bacilli when used against the European corn borer will be considered in Chap. 14.

ENTEROBACTERIACEAE INFECTIONS

The family Enterobacteriaceae is a large and important one, and its members are found throughout the animal and vegetable kingdoms in saprophytic and parasitic associations. Many of them live in the intestinal

tracts of animals, hence the name Enterobacteriaceae. As a group the enterobacteria are very homogeneous in morphology and in their fundamental biochemical characters. They are all gram-negative nonsporeforming rods. The family is divided into five tribes, of which members of the tribe Eschericheae are the most important from the standpoint of the diseases they cause in insects.

Coliform Infections

The tribe Eschericheae contains those gram-negative nochromogenic small rods which ferment lactose with visible gas formation within 1 or 2 days. These organisms are for the most part included in the genera *Escherichia*, *Aerobacter*, and *Klebsiella* and are frequently spoken of collectively as "coliform bacteria." (Some authorities consider these bacteria of common generic value and place them in a single genus, *e.g.*, *Colobactrum*.) Those which are characterized by consistently delayed fermentation of lactose (the so-called "slow lactose-fermenters") are frequently spoken of as "paracolon bacteria" and have been placed in the genus *Paracolobactrum*.

Now, within the coliform group are many biochemical types that in themselves do not constitute distinct species. Specialists in the group, therefore, have erected certain well-established criteria that serve as a nucleus about which these types or varieties may be arranged. These criteria have been centered in several well-known species: *Escherichia coli* (Mig.), *Escherichia freundii* (Braak), *Escherichia intermedium* (Werk. & Gill.), *Aerobacter aerogenes* (Kruse), and *Aerobacter cloacae* (Jordon). In other words, most of the gram-negative rods that rapidly ferment lactose will give biochemical reactions that, in general, approximate one or the other of the established species. Many of them will be close enough to these recognized species to be considered identical with them; others may be different enough to warrant their being considered as varieties. At the present time the nomenclatorial status of some of these varieties has not been clarified. Certain varieties were originally described as distinct species and still bear specific epithets. For example, *Escherichia paradoxa* (Toum.) (*Colibacillus paradoxus* Toum.) was isolated from diseased honeybees in France, and *Klebsiella capsulata* (Stern) was reported as causing an epizootic septicemia among bertha armyworms, *Barathra configurata* (Wlkr.), in North Dakota.

Aerobacter aerogenes and *Escherichia coli* both have been shown to be pathogenic when inoculated into the body cavity of certain insects. They are, however, common inhabitants of the intestinal tracts of many healthy insects. Even though they may normally occur in the intestines of an insect, these bacteria may be pathogenic when they gain entrance, natur-

ally or artificially, into the body cavity of the same insect. Both species have thus been reported experimentally pathogenic for the silkworm and other lepidopterous larvae. Larvae of the wax moth, *Galleria mellonella* (Linn.), are very susceptible to infection with *Escherichia coli* when this bacterium is inoculated directly into the insect; the latter usually dies within 12 to 24 hours.

Although the number of coliform bacteria isolated from diseased insects is probably larger than is now apparent—since the reactions of many of the entomogenous gram-negative nonsporeformers in modern differential media is unknown—comparatively few are noteworthy pathogens of insects. In fact, the significance of the entire group, as far as their pathogenicity for insects is concerned, is somewhat in doubt. To a considerable extent this is due to the uncertain taxonomic position of many, if not most, of the species described. The bacteria causing the diseases described in paragraphs to follow are treated here because, from the little information available, it seems likely that they are at least coliforms, even though their actual specific identities are in doubt or have not been generally recognized. Furthermore, as now conceived, certain bacteria responsible for several well-known diseases of insects actually belong to the coliform group even though they were originally placed in other genera. Since they illustrate some of the basic principles involved in bacterial infections, these diseases merit rather detailed consideration.

Dysentery and Septicemia of Grasshoppers

In 1910, while in Yucatan, Mexico, the bacteriologist d'Herelle observed an epizootic raging among locusts (*Schistocerca*) that had arrived in Mexico from the borders of Guatemala. The epizootic was so destructive to the insects that by 1912 the populations were reduced to such an extent that no invasion into Mexico occurred. From the dead or dying locusts d'Herelle (1911, 1912) isolated a small gram-negative rod, which he named *Coccobacillus acridiorum* and which he considered to be the cause of the disease. Many diseased specimens contained pure cultures of this bacterium. When inoculated into healthy locusts, the coccobacillus killed the insects with characteristic symptoms.

The apparent success of the disease in reducing grasshopper populations in Mexico created considerable excitement in other countries, and in 1911 d'Herelle was asked by the government of Argentina to try his methods in that country. Here, and in Colombia, he seemed to have local success in decreasing the number of grasshoppers. Success in Algeria was not so apparent, although somewhat satisfactory results were claimed in Tunisia where the disease was used in combination with mechanical methods. In the hands of other workers only dubious success was had in most cases.

The early hopes and enthusiasms gradually waned until finally the method was generally abandoned (see Chap. 14).

The Bacterium. Among the several explanations that have been given for the general lack of success of this method of control was that based on the belief that naturally occurring avirulent strains of the causative bacterium, as well as the presence of closely related bacteria, conferred an immunity on the insects and protected them from attack by the virulent bona fide "*Coccobacillus acridiorum.*" Furthermore bacteria were isolated which appeared morphologically and physiologically identical or similar to d'Herelle's organism but which were not pathogenic for the grasshoppers. Considerable confusion soon prevailed as to the exact role and significance of d'Herelle's organism in the dysenteric conditions occurring in grasshoppers in various parts of the world. Numerous strains, varieties, and species were circulated under the name "*Coccobacillus acridiorum*" (see Glaser, 1918*a*). The true identity of "*Coccobacillus acridiorum*" became confused, and its validity as a distinct species was questioned.

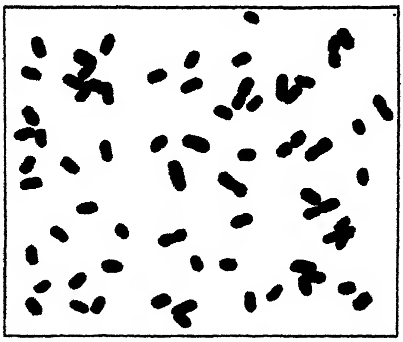


Fig. 87. *Aerobacter aerogenes* var. *acridiorum* (d'Her.) (= *Coccobacillus acridiorum* d'Her.), the cause of a bacterial dysentery in grasshoppers. (*After d'Herelle, 1914.*)

Recent studies (Steinhaus, 1948) of freshly isolated cultures, as well as of certain cultures isolated originally by d'Herelle, and critical examination and analyses of published data have presented convincing evidence to the effect that the so-called "*Coccobacillus acridiorum*" is in fact a coliform bacterium. Cultural and biochemical studies indicate that it is similar to *Aerobacter aerogenes* (Kruse) and *Aerobacter cloacae* (Jordon) and that it is very likely a variety of one of these species, probably *A. aerogenes.* However, since some strains ferment lactose rather slowly, it is conceivable that the bacterium may be related to *Paracolobactrum aerogenoides* B., S., & W. In any case, a designation such as *Aerobacter aerogenes* var. [or forma] *acridiorum* (d'Her.) com. nov. more accurately complies with rules of nomenclature than does the name *Coccobacillus acridiorum* d'Her. *Coccobacillus* is not recognized as a valid genus.

Aerobacter aerogenes var. *acridiorum* (d'Her.) is a gram-negative motile rod, many cultures showing both bacillary and ovoid forms. In young cultures and in the intestine of the grasshopper it frequently stains more heavily at the two extremities than in the middle. It grows well

on ordinary bacteriological culture media and ferments most of the ordinary sugars. Most strains apparently ferment lactose more slowly than does the typical *A. aerogenes*, although not so slowly as do most paracolons. The bacterium rapidly loses its virulence for grasshoppers with repeated transfers in artificial media. Its virulence may be enhanced by inoculation into and repeated passage through the host. The organism is not pathogenic for fowl, cows, sheep, or man, or for such laboratory animals as guinea pigs and rabbits, but the sewer rat dies in 3 or 4 days after a subcutaneous inoculation.

The bacterium which Glaser (1918*a*) named *Bacillus poncei*, and which he differentiated from "*Coccobacillus acridiorum*" on the basis of certain minor characters, is undoubtedly a coliform and appears to be a strain related to *Aerobacter aerogenes*.

The Hosts. Although d'Herelle originally reported the isolation of his coccobacillus from *Schistocerca pallens* Thunb.[1] in Yucatan numerous other species of grasshoppers have been reported as hosts to this bacterium. In Argentina d'Herelle directed his attack principally against *Schistocerca paranensis* Burm. In addition, he distributed the bacterium among an unidentified species of grasshopper of the genus *Caloptenus*, reporting complete control. In Colombia d'Herelle was concerned with the species known as *Schistocerca peregrina* Oliv., which he says is actually *Schistocerca americana* (Dru.). *S. peregrina* Oliv. was reported in Tunisia as being susceptible. In Algeria *Stauronotus maroccanus* Stål was similarly reported as a host, and the same grasshopper was partly controlled by the bacterium on the Isle of Cyprus.

Trials of other investigators have added several species to the host list. For example, Rorer (1915) reported the organism to be pathogenic for *Tropidacris dux* (Dru.), as well as for *Schistocerca parenensis* Burm. Glaser (1918*a*) found that a certain strain ("Souche Cham") of the bacterium was experimentally pathogenic for *Melanoplus mexicanus mexicanus* (Sauss.) (= *M. atlantis* Riley), and to a lesser degree for *Melanoplus bivittatus* (Say) and for *Melanoplus femur-rubrum* (DeG.). Another strain ("Souche Sidi") also appeared to be pathogenic for these species. DuPorte and Vanderleck (1917) added *Dissosteira carolina* (Linn.), *Camnula pellucida* Scudd., *Stenobothrus curtipennis* Scudd., and *Xiphidium* sp. to the list of experimentally susceptible hosts. A few other examples of experimental infection could be cited, and in all probability the list could be extended to great lengths, since experience has shown that nearly

[1] Some authorities are convinced that, although d'Herelle reported his original isolation for *Schistocerca pallens* Thunb., in actuality he was dealing with *Schistocerca paranensis* Burm. Others have thought he may have been concerned with *Schistocerca americana* (Dru.).

every species of grasshopper into the body of which adequate doses of the bacterium are artifically introduced, is susceptible to the septicemia thus produced. Natural infections have been observed in California in *Melanoplus differentialis* (Thos.) and *Schistocerca shoshone* (Thos.). Several species of ants and crickets have been reported as susceptible to the bacterium.

Among those grasshoppers which have been reported as being slightly or not at all susceptible to widespread destruction in epizootic proportions are *Locusta migratoroides* R. & F. (Mackie, 1913), *Zonocerus elegans* Thunb. (Lounsbury, 1913), and *Oedalens nigrofasciatus* DeG. (Barber and Jones, 1915).

Fig. 88. Grasshopper dead of bacterial dysentery. (*Courtesy of Ray F. Smith.*)

Symptoms of the Disease. The symptoms of the disease as it occurs in nature or as it results from experimental inoculation of the causative organism are essentially those which might be expected in dysenteric and septicemic conditions. After an incubation period of from 1 to 48 hours, depending upon the virulence of the bacterium, the individual resistance of the host, and on the weather conditions, the insect loses its appetite, becomes weakened, and moves in a faltering and ill-directed manner. Before long, the grasshopper ceases to feed altogether, becomes languid, is unable to jump, and seeks refuge under shrubs and other low vegetation. It may exhibit convulsive movements, moving its members, expecially the posterior legs, in a violent and spasmodic way, until it falls and is unable to right itself.

In the meantime, the contents of the gut become liquid, somewhat slimy in consistency, and blackish in color, and resemble agglutinated blood. Diarrhea usually begins shortly before the insect falls on its side and is left in a comatose state. By this time the bacteria have penetrated into the body cavity, causing a generalized septicemia and an invasion of the other tissues of the body. Infected gravid females are commonly unable to lay their eggs, and the latter are converted into a black mass. Sometimes the genital apparatus appears to atrophy. After a few minutes

or a few hours the animal dies, whereupon putrefaction sets in rapidly. The integument assumes a darkened color, and general decomposition follows.

Epizootiology of the Disease. Despite the considerable amount of attention given this disease of grasshoppers and the bacterium associated with it, the finer points of its epizootiology are far from being completely understood. In the first place there is much confusion as to the exact role of *Aerobacter aerogenes* var. *acridiorum* (*Coccobacillus acridiorum*) in the pathogenesis of the disease. Some investigators believe that perhaps the true cause of the infection may be an ultramicroscopic virus and that the bacterium is a rather consistent secondary invader. Some maintain that the bacterium is a true mutualistic symbiote invariably present in the insect, transmitted through the egg, and that either it is not capable of causing the disease, which must be due to other causes, or it occasionally "gets out of hand" and causes a morbid process in its host. Others are of the belief that the bacterium is a rather common inhabitant of the alimentary tract of grasshoppers and that under certain environmental conditions the organism becomes pathogenic for its host, and the characteristic disease results.

That bacteria indistinguishable from the "*Coccobacillus acridiorum*" of d'Herelle are rather consistently present in the alimentary tract of healthy grasshoppers of many species has been demonstrated repeatedly. Although in some cases rather constantly present, the bacteria are not, however, recoverable from every individual of a species, as might be expected if they were, in fact, true mutualistic symbiotes. Instead, they seem to be rather common commensals, ordinarily saprophytic. The data are not conclusive, but it would appear that, under circumstances that increase the susceptibility of the host or the virulence of the bacteria, the latter are capable of bringing about the disease we are discussing. The presence of any other agent as the primary cause of the disease remains to be demonstrated.

Just what the conditions are that favor the outbreak of the disease has also not been made entirely clear. It has been observed that rain and high humidity appear to "weaken" the grasshoppers, making them more subject to attack than they would be ordinarily. On the other hand, heavy rains apparently clean the foliage of the heavy doses of the bacteria left in the discharge of sick grasshoppers, and it is thought by some that this decreases the incidence of the disease. Low humidities, however, are unfavorable for the outbreak of the disease. Moderately cool to warm temperatures are favorable to the disease, although outbreaks do occur at high temperatures. The high ground temperatures that exist on prairie land appear to militate against the outbreak of an epizootic.

From what we have said concerning the identity and nature of the bacterium, it may be assumed that the organisms or closely related forms or strains are essentially world-wide in their distribution. Certainly we know that *Aerobacter aerogenes* itself is practically ubiquitous in nature, and it should not be surprising that the variety associated with grasshoppers is also widespread. Now it is not clear whether it is the bacteria already present in the insect that give rise to the infection or whether it is a particular strain or two that, after having acquired sufficient virulence, are distributed among the grasshopper population. That such distribution may occur is evident from the fact that the bacteria-containing discharges from diseased grasshoppers contaminate the foliage and food ingested by the insects. It is disconcerting, however, to find reports (*e.g.*, by Beltran, 1926) that experimental infection of grasshoppers by ingestion of bacteria scattered on foliage succeeds only exceptionally, although infection by injection into the body cavity is easily accomplished. In any case the bacterial flora of the grasshopper intestine is originally acquired perorally and if widespread transmission occurs, the oral route would appear to be the most logical. It would therefore appear that the cannibalistic habits of certain species of grasshoppers may be important in the transmission of the virulent bacteria. That this is the principal mode of transmission is the belief of many observers. The claims that the bacteria are transmitted via the egg need further substantiation with regard to any significance this might have in wide-scale epizootics.

The factors that appear to be important or necessary in influencing epizootics of the disease among grasshoppers, as conceived by various authors, may be summarized as follows: (1) appropriate weather conditions —high humidity and moderately warm temperatures, (2) dense populations, (3) cannibalistic and migratory habits of the host, (4) lack of immunity on the part of the host, and (5) no excess of normal food.

Use in Control. In spite of d'Herelle's original claims as to the efficacy of the bacteriological method of controlling grasshoppers, approximately 90 per cent of subsequent attempts made by other investigators in many parts of the world were either unsuccessful or only partly successful. Possible reasons for some of these failures are discussed in Chap. 14. The lack of attention to certain fundamental bacteriological principles by many of the experimenters undoubtedly accounts for a significant share of the failures. Even with expert handling, however, the pitfalls are so many that few agencies would care to devote the time and attention that the use of the bacterium concerned requires. The continual maintenance of the virulence of the cultures is in itself a matter that requires the greatest attention and skill. Probably the greatest single situation preventing the successful use of this bacterium as a control agent is our

gross ignorance of the many factors involved in its relation to its host and in the epizootiology of the disease it causes. Other aspects concerning the use of bacteria to destroy grasshoppers have been reviewed by Paillot (1933).

Other "Coccobacillus" Infections

Although the generic name *Coccobacillus* has no valid taxonomic standing, it has been used by a number of authors to include bacteria of the same general type as is the so-called "*Coccobacillus acridiorum.*" Like the latter organism, most of them are in reality coliform bacteria.

Shortly after d'Herelle made his first reports on "*Coccobacillus acridiorum,*" Picard and Blanc (1913*a,b*) observed that the larvae of the great tiger moth, *Arctia caja* (Linn.), once very abundant in the vineyards of southern France, were almost completely wiped out by two diseases. One disease was caused by a fungus, *Entomophthora aulicae* (Reich.), the other by a bacterium which they named *Coccobacillus cajae* Pic. & Blanc. The bacterium is a small motile gram-negative rod showing bipolar staining. It was isolated from the blood of the diseased caterpillars. The disease could be reproduced by either direct inoculation or by feeding the culture or other infectious material to healthy larvae. Septicemia developed within a few hours. In addition to the larvae of *Arctia,* the bacterium is experimentally pathogenic for several other Lepidoptera and for several species of Coleoptera. Aquatic beetles and several Hemiptera are among those insects which are immune. The fact that white rats are also immune, but a species of tree frog is susceptible, may be of interest to the student of comparative pathology.

In 1927, Metalnikov and Chorine (1928*a,b*) isolated a bacterium, which they called *Coccobacillus ellingeri,* from diseased larvae of the European corn borer, *Pyrausta nubilalis* (Hbn.). According to its discoverers, *Coccobacillus ellingeri* "somewhat resembles *Bacterium sphingidis* White, *Bacterium noctuarum* White, and *Bacillus melolonthae liquefaciens alpha.* It differs, however, from these three species by being non-motile." Since motility is a variable character, this would not be sufficient basis for establishing a new species. The published descriptions of these bacteria show further variations in their fermentation powers, but it appears likely that they are all members of the coliform group, since all have the general characteristics of this group.

Corn-borer larvae are very susceptible to infection by *Coccobacillus ellingeri* through the intestinal tract, the bacteria passing through the wall of the intestine into the blood where they are found in great numbers. Larvae infected in this manner die within 2 days. When inoculated directly into the body cavity with a small quantity of infectious material,

the larvae die in 2 to 12 hours; this applies as well to larvae of the wax moth, *Galleria mellonella* (Linn.). Guinea pigs and rabbits are apparently not susceptible.

Coccobacillus ellingeri M. & C. was one of four bacteria (the other three being *Vibrio leonardii* M. & C., *Bacterium galleriae* No. 2 M. & C., and an undescribed species) which Metalnikov and Chorine found would always produce mortal diseases of the corn-borer larvae when added to the food of the latter. Corn-borer larvae infected with *C. ellingeri* or *V. leonardii* keep their normal color for 2 or 3 days after death. *B. galleriae* No. 2 (which was originally isolated from diseased *Galleria mellonella* (Linn.)), on the other hand, turns the larvae black within a few hours after death, and there is rapid tissue destruction. With both *C. ellingeri* and *B. galleriae* No. 2 the rapidity with which the infection progresses accelerates with the increase in temperature. At 37°C. the larvae die within 1 or 2 days, while they live 2 to 6 days at laboratory temperature (19 to 21°C.). Virulence for peroral infection is rapidly lost with repeated transfers of the bacteria on artificial media. The virulence is maintained, however, if passages are made through larvae only. Metalnikov and Chorine made 86 consecutive passages in this manner with *C. ellingeri*, which retained its full virulence. The virulence of this bacterium varies considerably within a rather limited pH range. The optimum pH is 7.2. The greater the variance from this, on either side, the less virulent are the bacteria. This effect is apparently not one of difference in development of the bacterium, which from pH 6.5 to 7.6 does not vary to an appreciable degree. The addition of small amounts of sugars (0.5 to 1.0 per cent) in the culture medium appears to stimulate the virulence of the bacteria. (In certain other cases, as with *Bacillus thuringiensis* Berl., carbohydrates such as glucose appear to diminish the virulence.) The addition of egg yolk and horse serum to the medium yielded no such results.

According to Metalnikov and Chorine, the blood of insects shows a strong "oxygenophobia." It is rapidly oxidized in free air, turns black, and becomes very toxic for the insects. Insect blood contains very little oxygen, and the bacteria developing in the blood live more or less as anaerobes. Cultures of *C. ellingeri*, a facultative anaerobe, that had been grown under anaerobic conditions killed 83.0 per cent of the corn-borer larvae; those grown with free access to oxygen killed 71.4 per cent. The corresponding figures for *B. galleriae* No. 2 were 84.6 per cent and 62.6 per cent. It would be interesting to know to what extent this difference may be apparent in the case of other entomogenous bacteria.

Coccobacillus gibsoni Chor. is another bacterium that is pathogenic for larvae of the European corn borer both by injection and by feeding it to the insect. Chorine (1929*a*,*b*) isolated the bacterium from disease

larvae received by him from Canada. It is a polymorphic gram-negative small rod, producing acid and gas in glucose and other carbohydrates. Acid and some gas is produced in lactose, which indicates the coliform nature of the bacterium.

Coccobacillus insectorum var. *malacosomae* Holl. & Ver. is the name Hollande and Vernier (1920) gave to a coliform bacterium they found causing a septicemia of the tent caterpillar, *Malacosoma castrensis* Linn. *M. neustrium* (Linn.) and *Vanessa urticae* (Linn.) are also susceptible to the same organism.

Hornworm Septicemia

As we have pointed out elsewhere, the term "septicemia" as the name of a disease is perhaps undesirable since it has no specific connotation. In the case at hand, for example, hornworm septicemia refers to the disease in hornworms caused by *Bacterium sphingidis* (White), yet a septicemia in this insect caused by any other bacterium could be equally well designated by the term "hornworm septicemia." As yet, however, no satisfactory system of nomenclature for the diseases of insects has been devised, and until such a system exists much of the terminology in insect pathology will remain unsatisfactory. Since the terms "hornworm septicemia" and "cutworm septicemia" are used as such in most of the literature, we shall provisionally use them in the discussions to follow.

Hornworm septicemia is a disease first described by White (1923*a*) in the larvae of two species of insects, *Protoparce sexta* (Johan.) (tobacco hornworm) and *Protoparce quinquemaculata* (Haw.) (tomato hornworm), in which during the course of the infection the bacterium enters the body cavity and multiplies rapidly in the blood. We shall discuss this disease in some detail, since in its symptoms, pathology, and general characteristics it typifies the septicemic conditions caused by numerous species of bacteria in most caterpillars.

Very few references to diseases in hornworms exist prior to White's report, and these may or may not be the same infection with which he worked. Dead and blackened hornworm larvae hanging head down from plants were reported in 1897, and a "bacterial disease" of these insects was reported in 1915. In 1917, employees at a Federal tobacco insect laboratory in Tennessee encountered the disease under consideration in their experimental colonies and observed that it could be transmitted to healthy larvae by the puncture method of inoculation. White (1923*a*) obtained the material for his studies from this laboratory, and his report is the source of most of our knowledge of the disease.

Symptoms and Post-mortem Changes. Hornworm larvae suffering from the disease lose their appetite and become sluggish in movement.

The normal stool of berrylike pellets changes in the infected insects to a semifluid one and then to a watery discharge. Later, a thin vomit oozes from the mouth. The pronounced turgidity of healthy larvae is not present in the infected ones. On a flat surface the insect usually dies lying on its side in a slightly curved position. When the insect dies on its host plant, the remains are usually found hanging head downward by means of the hooks of a proleg. When in this position, the semifluid body content gravitates toward the head end.

Fig. 89. Hornworm (*Protoparce*) 2 days after inoculation with *Bacterium sphingidis* (White), hanging by a proleg from leaf of tobacco plant on which it had been feeding. (*From White, 1923a; courtesy of U.S. Department of Agriculture.*)

Shortly after the hornworm dies its body becomes light brown in color, deepens rapidly to a dark shade, and finally turns almost black. The body wall at first resists puncture and tearing to about the same degree as that exhibited by living larvae, but later it can be ruptured rather easily. The internal tissues undergo a rapid change, becoming a brown semifluid mass in which silvery-white portions of tracheae may be seen. Ordinarily, when the decaying larva is not disturbed, the body wall remains intact. As drying takes place, the remains diminish in size although they retain the general larval form. In a week or so the hornworm is a dry, shriveled, friable, dark brown to black mass.

The histopathology of the disease has not had detailed study. Microtome sections of the sick larvae, however, show that the infection is primarily a septicemia. The bacteria may be seen throughout the blood spaces of the insect's body. They are especially numerous between the folds of the stomach wall.

The Causative Bacterium. The bacterium causing hornworm septicemia was originally named *Bacillus sphingidis* by White (1923*a*) and at one time or another has also been placed in the genera *Escherichia* and *Proteus*. That it does not belong in the sporeforming genus *Bacillus* is apparent since it is a gram-negative nonsporeforming small rod, approximately 0.5 to 0.7 by 0.8 to 1.5 microns in size. Coccoid, ovoid, and filamentous forms also occur. It is motile, most frequently possessing one flagellum, but occasionally two or more flagella, usually attached near an end of the rod. Acid and a small amount of gas are formed in glucose, but in lactose only a slight amount of acid is produced in 24 hours, followed by a change to alkalinity. These fermentation characteristics indicate

that the organism is probably a paracolon bacterium. In the absence of further study, it is probably just as well to consider the organism as inadequately classified and tentatively to designate it as *Bacterium sphingidis* (White.)

In all probability this bacterium is one that is ordinarily saprophytic, becoming pathogenic for insects under certain environmental conditions. Warm weather, for example, seems to predispose the hornworm to the septicemia, and infection appears more likely to occur during the fifth instar. White found there to be no appreciable loss of virulence in cultures kept on artificial media for 4 years.

Pathogenicity of the Bacterium. The inoculation of infectious material into the body cavity of healthy hornworm larvae results in a mortality of 100 per cent. When infectious material is fed to the larvae, a mortality of only about 10 per cent results. Hornworms of all instars are very susceptible to infection with pure cultures of *Bacterium sphingidis* when the puncture method of inoculation is used. Infectious material direct from the diseased larvae usually kills the insects slightly faster than do cultures of the bacterium.

The period of time from the inoculation of a hornworm by puncture to its death varies considerably, depending chiefly on the temperature. During warm summer days, death takes place in 1 day or less; in cooler weather the time is extended to 2 or more days. Once the bacterium enters and starts multiplying in the blood of the insect, death is an almost inevitable outcome. Some phagocytosis occurs, but this usually does not offer sufficient protection to save the larva.

In addition to hornworms, certain other insects are known to be susceptible to *Bacterium sphingidis*. These include such insects as the silkworm, larvae of the catalpa moth, cutworms, and grasshoppers. Experimental vertebrate animals (*e.g.*, rabbits) do not appear to be susceptible to the bacterium when inoculated intravenously.

The manner in which the disease is transmitted in nature is not known; neither is its distribution. Wide-scale epizootics in the field probably occur only rarely, and the artificial use of the bacterium in the control of hornworms is apparently not practical.

Cutworm Septicemia

Correctly speaking, the designation "cutworm septicemia" might refer to any of a number of body-cavity infections in cutworms. The term was specifically used, however, by White (1923*b*) to describe an infection encountered among cutworms (*Feltia*) at the Bureau of Entomology laboratory in Tennessee. In most of its general characteristics this cutworm septicemia is similar to the hornworm septicemia just described.

Symptoms and Pathology. The symptoms of cutworm septicemia are essentially the same as those of hornworm septicemia. The affected insects have a failing appetite, are listless, lack the normal turgidity of the body, and have a diarrhea, and there is usually a thin discharge from the mouth. Death ordinarily takes place within 2 to 4 days after onset, whereupon the color of the flaccid insect changes to a brown, which deepens as the disintegrative processes continue. The internal tissues become a thick brown nonviscid mass, which becomes brittle upon drying. There is a slight but not disagreeable accompanying odor.

Histological sections of the diseased larvae show bacteria present in the blood spaces and in the stomach near the epithelium. Phagocytosis apparently occurs to some extent, and there is an increase in the number of blood cells.

The Causative Bacterium. The bacterium causing the cutworm septicemia under discussion is very closely related to that responsible for the hornworm septicemia. It is a gram-negative nonsporeforming short rod, probably belonging to the paracolon group of bacteria. The only significant difference that White claimed to have found between this bacterium and that of the hornworm disease is a serological one. This difference may not, however, be great enough to distinguish them as separate and distinct species. Both these bacteria appear to be rather closely related to "*Coccobacillus acridiorum*," and all three organisms probably belong to the same general category.

White (1923*b*) originally gave to the cutworm bacterium the name *Bacillus noctuarum*. At different times "Bergey's Manual" has placed it in the genera *Escherichia* and *Proteus*. Until the true taxonomic status of this organism is determined it is perhaps most convenient to refer to it tentatively by the designation *Bacterium noctuarum* (White) or else to consider it synonymous with *Bacterium sphingindis* (White).

The manner in which *B. noctuarum* is transmitted from one insect to another is not clear. Infection probably follows the ingestion of a sufficient dose of the bacteria, although experimentally the oral route is not a very effective one, especially when compared with puncture inoculations, which result in 100 per cent mortalities. The low susceptibility of cutworms to ingested bacteria probably is related to the fact that extensive infections in the field rarely occur. As with hornworm septicemia, the disease in cutworms may be enhanced by warm temperatures.

Silkworms, catalpa-moth larvae, grasshoppers, and probably numerous other insects are susceptible to *B. noctuarum* when inoculated by the puncture method. Rabbits, and probably other vertebrates, are not susceptible.

The literature contains several references to other "septicemic" conditions in cutworms. In some cases the causative bacterium was not de-

scribed. For example, in 1899 Cavara observed such a disease in Italy among *Agrotis aquilina* Hbn. The small rod-shaped bacterium he isolated from the diseased insects may have been similar to that described by White. Experimentally it is also pathogenic for *Hylotoma pagana* Panz. when inoculated by puncture but not when inoculated by feeding. In 1927 Pospelov, in Russia, described a septicemia of *Euxoa segetum* Schiff. caused by a gram-negative nonsporeforming rod he called *Bacillus agrotidis typhoides*. He observed the disease in nature during the autumn and spring. From other larvae of the same species of cutworm, Pospelov isolated "*Bacillus nonliquefaciens* (*putidus* Flügge) and *Bacillus fluorescens liquefaciens* Flügge." These larvae also had symptoms of a septicemia. Bacterial septicemias frequently accompany certain virus infections. In these cases the bacteria are usually secondary invaders.

Potato-beetle Septicemia

The name "potato-beetle septicemia" was used by White (1928, 1935) to describe an infection in larvae of the Colorado potato beetle, *Leptinotarsa decemlineata* (Say), caused by a bacterium similar to those responsible for the septicemias just described in the hornworms and cutworms. In this case the name of the causative organism is *Bacterium leptinotarsae* (White) (formerly *Bacillus leptinotarsae* White). Like the others, it is a small gram-negative motile rod, growing well on the usual bacteriological media. Since the organism ferments lactose with the production of a slight and transient amount of acid, it probably belongs to the paracolon group of the tribe Eschericheae. White (1935) occasionally isolated *B. leptinotarsae* from apparently healthy larvae, and from the diseased insects he isolated other species of bacteria that were nonpathogenic. Cultures of the feces of healthy larvae ordinarily yielded only a few colonies, usually of chromogenic bacteria (see also Steinhaus, 1941).

Potato-beetle larvae affected with the septicemia are at first sluggish and then soon become motionless. They lose their appetite and cease to feed. When moribund or dead they fall to the ground, although occasionally dead specimens are found adhering to the food plant. Soon after death the reddish tint of healthy larvae changes to a brownish-gray; later the color becomes dark brown or nearly black. In about a week, after drying takes place, the remains are a shriveled dark brittle mass. Experimentally, other insects (hornworms, cutworms, silkworms) are susceptible to inoculations of the bacterium, and for the most part they show symptoms more or less typical of septicemia.

It appears possible that *B. leptinotarsae* may be one of the factors in the natural control of the Colorado potato beetle. Natural outbreaks of the disease apparently are not infrequent. Its importance in artificial-

control measures remains to be demonstrated. The fact that White found the organism not to be readily transmitted through feeding inoculations would make the spraying of potato plants with suspensions of the bacterium seem impractical on the basis of present information. It is possible that certain predisposing causes are among the factors favoring the disease.

Serratia Infections

Three species of *Serratia* (tribe Serrateae) have been recorded as being associated with insects, but only one, *Serratia marcescens* Bizio, has been considered as at times a pathogen of insects. *Serratia kilensis* (L. & N.) (= "*S. kielensis*") has been used to demonstrate the ability of flies to transmit bacteria (Cao, 1906*a*,*b*). Some of these experimental flies died earlier than usual, and it is possible that the bacterium may have been responsible for these deaths. The third species, *Serratia plymuthicum* (L. & N.) (= *S. plymouthensis* (Mig.)), was isolated from healthy crickets collected in nature in Ohio (Steinhaus, 1941). It appeared to be a normal inhabitant of the insect's gut and caused no pathogenic effects.

The genus *Serratia* consists of small gram-negative rods, which usually produce a characteristic red or pink pigment, although white to rose-red strains frequently occur. Ordinarily they are common saprophytes, found in such habitats as water, soil, and milk and other foods. They are nonpathogenic to vertebrates except in enormous doses.

Serratia marcescens, the species that concerns us here, is also known by several synonyms, including *Bacillus prodigiosus* Flügge, *Bacterium prodigiosum* L. & N., and *Chromobacterium prodigiosum* Top. & Wil. Much of the earlier literature on this organism uses one or another of these synonyms. The priority of the name *Serratia marcescens*, however, is now well established.

Insect Diseases Caused by *Serratia marcescens*. As early as 1817, Rozier noticed a red coloration forming in the bodies of dead silkworms (*Bombyx mori* Linn.). This phenomenon was again noticed by Pollini in 1819, by Re and by Ascolese in 1837, and by several other early observers. The credit for the actual isolation of *Serratia marcescens* from such silkworm larvae apparently belongs to Perroncito, who accomplished this in 1886. About the same time Bandelli isolated the bacterium from the exterior of silkworm larvae and later wrote that the red pigment does not appear until after the death of the insect.

Between 1934 and 1936, the Italian worker Masera published a series of notes in which he recorded the results of his experiments on the pathogenic action of *S. marcescens* toward a number of insects, including the silkworm. In general he found that the bacterium almost always produces a fatal

infection when inoculated into the silkworm but that, when introduced along with the insect's food, it frequently does not kill the host and that, when given by ingestion, it is pathogenic in varying degrees according to the stage of the larva. The percentage of deaths is higher the nearer the insect is to the pupal stage. Several other investigators have reported the general nonsusceptibility of the normal silkworm to the organism by ingestion, and still others have obtained contrary results.

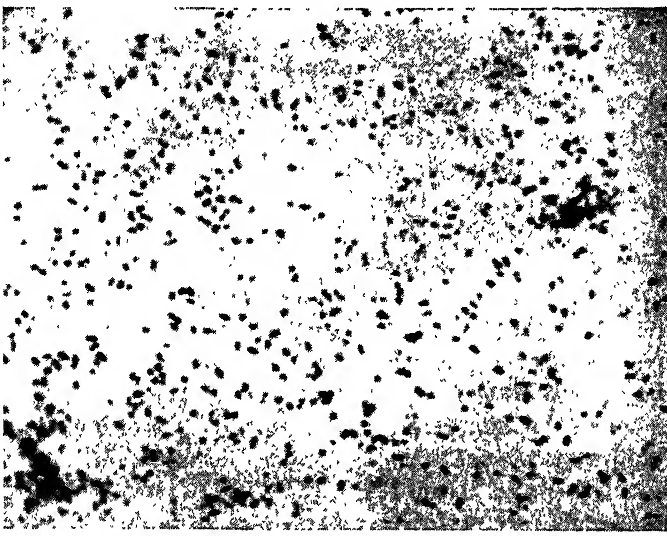


Fig. 90. Stained smear of *Serratia marcescens* Bizio, a red-pigmented bacterium which, under certain conditions, is capable of causing infections in insects. (*From "Insect Microbiology," Comstock Publishing Company, Inc. Photograph by N. J. Kramis.*)

Under insectary conditions, the bacterium is found associated with the silkworm primarily when the caterpillar is infected with the fungus *Beauveria bassiana* (Bals.). The exact relationship between the bacterium and the fungus is still not very clear. Masera observed that on artificial media there is a definite antibiotic effect exhibited by the bacterium against the development of the fungus. He was able to demonstrate this same phenomenon in the larva of the yellow mealworm, *Tenebrio molitor* Linn., which is not susceptible to the pathogenic action of the bacterium, especially when the latter is given perorally. The bacterium appeared to reduce the incidence of infection by the fungus in a colony of these insects. In the case of the silkworm, Masera believes that the caterpillar is made more susceptible to the fungus through the action of *S. marcescens*.

In addition to the silkworm, a number of other insects are known to be susceptible to *S. marcescens*, naturally as well as experimentally. Larvae of the wax moth, *Galleria mellonella* (Linn.), are susceptible to the bacterium

by injection but not by ingestion. The European corn borer, *Pyrausta nubilalis* (Hbn.), and larvae of the gypsy moth, *Porthetria dispar* (Linn.), are susceptible by both routes. Also experimentally susceptible are the beet webworm, *Loxostege sticticalis* (Linn.); the cabbage butterfly of Europe, *Pieris brassicae* (Linn.); *Hyponomenta malinella* Zell.; and the white-fringed beetle, *Pantomorus peregrinus* Buch. Attempts to control some of these insects, particularly *L. sticticalis* (Linn.), with *S. marcescens*, though promising in the laboratory, have not yielded very satisfactory results in the field (Drobotjko, Martshouk, Eisenman, and Sirotskaya, 1938). Termites (*Zoötermopsis*) may also be susceptible to the bacterium.

Outbreaks of infection caused by *S. marcescens* occur not infrequently in laboratory-reared and insectary-reared insects. In 1937 Lepesme reported such an outbreak in his colony of *Schistocerca gregaria* Forsk. The disease was essentially a septicemia. When infected with the bacterium, the locusts usually die within 1 or 2 days, their abdomens assuming a characteristic red color. Ingestion of the bacterium only occasionally results in the death of the host. The last nymphal instar appears to be more susceptible to infection than do the preceding ones. The bacterium is also known to be a secondary invader to an infection in the locust caused by the fungus *Aspergillus flavus* Link. Spraying cultures of the bacterium on host plants in the field may result in the infection of some locusts, but the possibilities of wide-scale control with this organism do not appear to be great (Barber and Jones, 1915).

The potato tuberworm, *Gnorimoschema operculella* (Zell.), is also subject to infection by *S. marcescens*, especially when it is being reared in large numbers (Steinhaus, 1945). Infected individuals become sluggish in movement, less sensitive to external stimuli, have a lessened appetite, and frequently are diarrheic. Upon death, the larvae become bright red in color, turning brown upon subsequent disintegration. Occasionally, infected larvae are capable of going into the pupal stage, after which they assume the red color of diseased individuals. Of particular interest, from a bacteriological viewpoint, is the fact that, in the investigations just referred to, none of the strains of *S. marcescens* isolated showed the dissociation into the pink and white colors usually seen in this species upon subsequent transfers. Insect parasites, such as *Macrocentrus ancylivorus* Roh. and *Dibrachys cavus* Wlk., reared on tuberworms frequently acquire the infection from the host insects.

Since *S. marcescens* occurs commonly in nature, it is not surprising that individual insects are from time to time found infected with this bacterium. Sometimes, as has been the case with a beet weevil in Russia, the infection is found in considerable numbers of a given species in one general area. Extensive epizootics, however, are apparently very rare.

Some insects, such as the honeybee, *Apis mellifera* Linn., and the yellow mealworm, *Tenebrio molitor* Linn., have been reported as being insusceptible when fed the bacterium. In the case of the latter insect, Masera (1934*b*,*c*) believes that the insusceptibility may be accounted for by an immunity produced in the insects as a result of previous contact with the bacterium in the insect's food. Other experiments have indicated that the resistance of the insects is not explained on such a coincidental basis.

Other Enterobacteriaceae Infections

A few species of bacteria in the remaining tribes and genera of Enterobacteriaceae have been reported as pathogenic for insects. None of these, however, are what may be considered as classical insect pathogens.

Salmonelloses of the adult honeybee have been reported by Bahr (1919) and Toumanoff (1939). Bahr discovered bees in the vicinity of Copenhagen suffering from an acute enteritis caused by a small asporogenic rod he designated as *Salmonella schottmuelleri* var. *alvei* Haud. The infected bees usually died in from 25 hours to a few days. Certain of the strains isolated by Toumanoff in France from diseased bees were similar to that described by Bahr and were characterized as belonging to the salmonella, or paratyphoid group, of bacteria. The intestinal tract of the infected bees is usually packed with the organisms. The peritrophic membrane is destroyed, and there is some penetration of the epithelial cells by the bacteria. Histologically the cells assume certain abnormal aspects. The nuclei present signs of disintegration, and the protoplasm stains more feebly than normally. The intestinal contents are characteristically fluid in consistency.

Some insects, as well as ticks, have been shown to be susceptible to certain *Salmonella* when the bacteria are inoculated experimentally. Larvae of the wax moth, *Galleria mellonella* (Linn.), have been thus infected with *Salmonella schottmuelleri* (Winslow) and with *Salmonella enteritidis* (Gaer.). The latter organism may be fatal to *Dermacentor andersoni* Stiles when fed to this tick. In addition, strains of *Salmonella* have been found associated with filth-frequenting insects, such as flies, but such associations are usually purely fortuitous in character. The same may be said of the typhoid bacillus, *Salmonella typhosa* (Zopf) (= *Eberthella typhosa* (Zopf)), which is well known in its relation to and distribution by flies. Some workers have found larvae of the wax moth to be experimentally susceptible to the typhoid bacillus, while others have found no pathogenicity involved. The same inconsistency has been reported in the case of *Shigella dysenteriae* (Shiga), the cause of bacillary dysentery in man.

Several species of *Proteus* have been described as causing diseases in

insects, but none of these has been included as a bona fide species in recent revisions of the genus (*e.g.*, that by Rustigian and Stuart, 1945). Although these omissions probably occurred through oversight or because cultures of these species were not available for study, it is probable that some of the species concerned do not, in fact, belong in the genus. *Proteus alveicola* Serb., for example, was described by Serbinow (1915) as the cause of a diarrhea of the honeybee, in association with "*Bacterium coli apium.*" *Proteus bombycis* Bergey *et al.* was originally isolated and described by Glaser (1924), who, however, did not name the organism. The name appears to have been provided by the editors of "Bergey's Manual" (3d ed.) and is now believed to be a strain of *Paracolobactrum aerogenoides* B., S., & W. Lehmann gave the name *Bacterium bombycivorum* to the same bacterium. The organism was isolated from the feces, blood, and various tissues of diseased silkworms. Normal caterpillars could be infected by feeding them food contaminated with the feces and body fluids of diseased worms.

The members of the genus *Erwinia* are generally regarded as plant pathogens. Needham (1937), however, isolated from diseased *Aphis rumicis* Linn. an organism closely resembling *Erwinia lathyri* (Manns. & Taub.). This bacterium was originally isolated from sweet peas.

BACTERIACEAE INFECTIONS

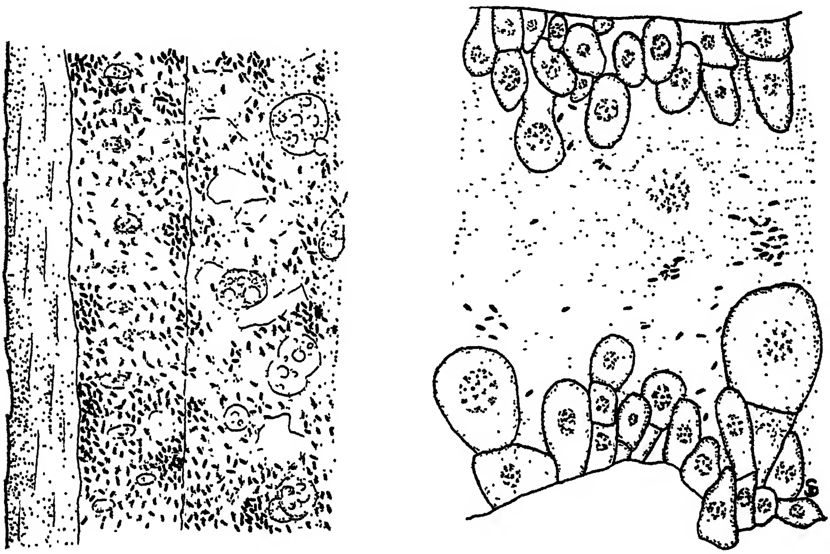
According to "Bergey's Manual," the family Bacteriaceae is, for the most part, a rather "heterogeneous collection of genera whose relationships to each other and to other groups are not clear." In this family the generic name *Bacterium* "is used to cover species of non-spore-forming, rod-shaped bacteria whose position in the system of classification is not definitely established." Certainly a considerable number of bacteria described from insects could come under this characterization. As we have already pointed out, from the general characteristics given in their published descriptions, certain species can be removed from this group and placed with the coliform bacteria. This we have done to the extent that we have been able to on the basis of available information. A few entomogenous species of *Bacterium* appear to defy definite classification on the basis of their published descriptions, but at least one of these and the disease it causes are worth consideration here.

Toxic Septicemia of the Squash Bug

In July, 1895, Duggar observed squash bugs (*Anasa tristis* DeG.), which he was keeping in rearing cages, dying in considerable numbers. He propagated the infection among fresh insects and finally isolated the

bacterium responsible for the disease. This bacterium is an interesting one because of the peculiar toxic effects it produces in insects.

Symptoms. As described by Duggar (1896), a few hours before death the infected insect may be found in a sluggish condition, resting low on its ventral surface and often apparently incapable of raising itself erect or of crawling without a marked drag. If placed on its back, it has no



A *B*

Fig. 91. Infection of the tissues of the squash bug, *Anasa tristis* DeG., by *Bacterium entomotoxicon* (Duggar). *A*. Distribution of the bacteria in the hypodermis and adipose tissue of a squash bug at the time of death. *B*. Longitudinal section of the dorsal vessel of a squash bug slightly sick, showing distribution of the bacteria in the blood. (*Redrawn from Duggar, 1896.*)

power to return to its normal position. As the disease progresses the insect loses nearly all muscular activity, and a slight waving of limbs and antennae may be the only indication of life. Although they usually frequent the undersurface of the leaf, normal squash bugs cannot attach themselves strongly to the plant by their limbs. For this reason, when diseased bugs lose their grip, they commonly drop to the ground where they are found dead or dying. Although there is no marked discoloration of the body some hours before death, the diseased insect becomes slightly darker as death approaches. After death, disintegrative changes take place rapidly, and the bacteria may be found in large numbers in the blood and tissues.

Diseased nymphs take on a deep purplish-black coloration. The body

undergoes no shrinkage but appears tense and slightly swollen. Within 24 hours or more it becomes a mere sac filled with a dark-colored fluid. In this condition the body wall readily collapses, and the insect frequently disintegrates when an attempt is made to lift or remove it.

Adult squash bugs have a rather moist appearance at the time of death, especially in the anterior region of the ventral surface of the abdomen. The wet appearance subsequently becomes apparent over the entire body. The hard chitinous layer does not shrink or collapse, and the offensive-smelling fluids within go unnoticed unless the wall is broken. The odor characteristic of the disease is more pronounced than is the normal squash-bug odor. Shortly after death the appendages may be separated with ease at the articulations, and it is almost impossible to lift the insect by means of them.

The Bacterium and Its Pathogenic Properties. Although Duggar originally gave to the organism concerned the name *Bacillus entomotoxicon,* the fact that he described it as a short nonsporeforming rod would probably make the designation *Bacterium entomotoxicon* (Duggar) more applicable for the time being. Its reaction to the Gram stain was not determined by Duggar; apparently it is gram-negative. With simple stains it has a bipolar appearance. On nutrient agar, the organism forms a dirty-white colony often characterized by prominent fanlike radiations. The latter characteristic, as exemplified by Duggar's illustration of it, is suggestive of the colony form of certain sporeformers.

The bacterium apparently liberates a toxic substance that will rapidly kill specimens of several species of insects when they are immersed in broth infusions of the organism. In addition to squash bugs, it has been observed that chinch bugs, flies, and certain other insects react to the action of this toxic substance, although there are indications that grubs and lepidopterous larvae are refractory. When an insect is immersed in this toxic broth, it soon becomes sluggish and in a few minutes makes characteristic incoherent movements. After about 5 minutes the insect stiffens and becomes rigid, and the legs are closely drawn together. Duggar referred to this phenomenon as "toxic rigor." Insects will frequently recover if removed from the broth as soon as they become stiffened. The effect is not one of a drowning, since even water beetles rapidly succumb to it. The nature of this substance, however, is not known.

Histological sections of diseased squash bugs show that the bacterium is present in the blood at all stages of the disease. The hypodermis, adipose tissue, and cardiac tissue are also infected early in the course of the disease. Cultures of the diseased insects rarely yield saprophytic organisms, and in most cases pure cultures of *Bacterium entomotoxicon* are obtained.

LACTOBACTERIACEAE INFECTIONS

The family Lactobacteriaceae consists of two tribes: (1) Streptococcaceae, which includes gram-positive cocci occurring in pairs and chains; and (2) Lactobacilleae, which includes gram-positive rods producing lactic acid from carbohydrates. None of the latter cause diseases in insects; we shall therefore limit ourselves to the tribe Streptococcaceae, which contains three genera: *Diplococcus, Streptococcus,* and *Leuconostoc.* The last-named genus has not been found causing infections in insects.

Diplococcus Infections. The differentiation between *Diplococcus* (cocci in pairs) and *Streptococcus* (cocci in chains) is not always clear or easy. Probably most of those bacteria associated with insects which have been described as *Diplococcus* actually belong to another group, such as *Streptococcus,* which under certain circumstances occurs in pairs or in very short chains.

From diseased silkworms, *Bombyx mori* (Linn.), Paillot (1922) isolated a "coccobacillus" which he called *Diplococcus bombycis.* The organism is an elongated gram-positive coccus and characteristically shows transverse double bands of chromatin material. The larvae of *Porthetria dispar* (Linn.) and *Nygmia phaeorrhoea* (Donov.) appear to be very resistant to infection with the bacterium, while the larvae of *Eriogaster lanestria* Linn. and *Vanessa urticae* (Linn.) may be infected rather easily. Earlier, Paillot (1917) had isolated both *Diplococcus liparis* Paill. and *Diplococcus lymantriae* Paill. from the larvae of the gypsy moth, but neither of these bacteria seems to be a very important pathogen. He also found that an organism he called *Diplococcus melolonthae* was only slightly pathogenic for cockchafers, *Melolontha melolontha* (Linn.), when used alone, but when inoculated along with a certain unidentified "coccobacillus" the insects appeared to be more susceptible to the diplococcus. *Diplococcus pieris* Paill. has been found in the cabbage butterfly, *Pieris brassicae* (Linn.), as a secondary invader to the hymenopteran parasite *Apanteles glomeratus* (Linn.).

Several investigators have observed *Diplococcus pneumoniae* Weich., the cause of pneumonia in man, to be nonpathogenic to larvae of the wax moth, *Galleria mellonella* (Linn). This is another example of the low pathogenicity of vertebrate pathogens for insects.

Streptococcus Infections. Two of the best known entomogenous streptococci are *Streptococcus apis* Maassen, associated with European foulbrood, and *Streptococcus bombycis* auctt. (= *Micrococcus bombycis* Cohn), associated with gattine. *S. apis* has been considered earlier in the chapter in our discussion of the etiology of European foulbrood. Some authorities believe that this organism is in reality a variety or stage

in the life history of *Bacillus alvei* Ches. & Chey., generally recognized as the exciting cause of the disease. Others consider it to be a synonym of *Streptococcus liquefaciens* Stern.

Streptococcus bombycis was for a long time believed to be the cause of gattine. It now appears that the primary cause of this disease of silkworms is a virus and that *S. bombycis* is associated with the disease as a rather constant secondary invader. For this reason, gattine will be more fully described when we discuss the viruses affecting insects. *S. bombycis* is a gram-positive coccus forming chains usually from 4 to 12 members long and is a facultative anaerobe. In gattine, this organism may be isolated from the intestinal contents of the diseased silkworms.

Another streptococcus that has had detailed study is *Streptococcus disparis* Glaser, which causes an infectious disease of the caterpillars of the gypsy moth, *Porthetria dispar* (Linn.). This bacterium was isolated and described by Glaser (1918*b*) after the disease first broke out in a Japanese race of caged gypsy-moth larvae and spread to specimens of the American race. Glaser had provisionally called it the "Japanese gypsy-moth disease." It is a gram-positive capsulated coccus usually arranged in chains of three or four units. It is nonmotile, as are most cocci, and it grows on ordinary bacteriological media such as nutrient agar, does not liquefy gelatin, and ferments carbohydrate media with the production of acid.

The symptoms of the disease in gypsy-moth caterpillars begin with a steady loss of the appetite until the insect ceases to eat, and with a rather violent form of diarrhea. The diarrheic discharges are filled with streptococci, and transmission probably is aided by the soiling of the food plants with the semifluid feces. The larva seems to lose all muscular coordination and usually crawls slowly to some elevated place, where it soon dies. After death it hangs by its prolegs in a flaccid manner. Although the general appearance of the insect may simulate that of the polyhedrosis in this insect, it may be distinguished grossly from the virus disease by the fact that one can pick up and stretch the animal with considerable force before the skin breaks; virus-infected insects are usually much more fragile. Experimentally, the period from infection by feeding to death varies considerably. Death may occur any time after 24 hours and may be postponed for as long as 16 days. This variation probably depends on differences in the number of bacteria ingested and on individual resistance or immunity. *S. disparis* is not pathogenic for silkworms, certain army-worms, guinea pigs, rabbits, and human beings.

During the early stages of the disease the streptococcus is found throughout the alimentary tract of the host. Later, and particularly after death, the intestinal epithelium disintegrates and ruptures, liberating

Fig. 92. The effect of infection by *Streptococcus disparis* Glaser on the muscle tissue of the gypsy moth, *Porthetria dispar* (Linn.), shown diagrammatically and photographically. *A*. Normal muscle tissues. *B*. Muscle tissue rather late in the infection showing the separation of the fibrillae. *C*. Last stage in the pathology of gypsy-moth muscle tissue, showing complete degeneration. *D–F*. Photographs showing changes more or less comparable with those diagramed. (*Photographs from Glaser, 1918b.*)

the bacteria into the body cavity. An invasion of all the tissues follows. Marked changes occur in the muscle tissues during the course of the disease. In the earliest stages of the infection histological sections show that the striae are losing some of their definiteness, and the individual fibrillae seem to be more loosely arranged. Later the muscle tissue loses its striated appearance because the fibrillae have lost their compactness and have separated from one another "like threads of cotton." Along with the rest of the tissue the sarcolemma gradually disintegrates, and the nuclei of the cells assume abnormal positions and become scattered. Eventually complete disintegration of the muscle tissue takes place, the fibrillae no longer remain visible, and the entire tissue resembles coagulated protein material throughout which minute granules are scattered. By the time the muscles have disintegrated to this extent, the other tissues of the host have also broken down more or less, and the streptococci are scattered throughout the insect's body.

From the standpoint of microbial control, the over-all importance of *S. disparis* apparently is not great. Glaser did report that field trials in two localities in Massachusetts were successful in producing significant epizootics among gypsy-moth caterpillars. No great difficulty was had in reproducing the disease in the field, but its natural presence in gypsy-moth populations could not be substantiated.

The type of muscle disintegration characteristic of *S. disparis* infection in gypsy-moth larvae is also seen in a similar infection of one of the processionary moth caterpillars, *Thaumetopoea pityocampa* (D. & S.) (= *Cnethocampa pityocampae* D. & S.) caused by *Streptococcus pityocampae* Duf. Dufrenoy (1919) described two varieties (*alpha* and *beta*) of this organism, but there is reason to question the generic location he gave them. The *alpha* strain is motile, and the *beta* strain is gram-negative, characteristics not ordinarily ascribed to the genus *Streptococcus*.

Streptococcus pyogenes Rosenb., the cause of certain types of streptococcus infection in man, has been isolated from normal flies but has never been found causing an infection in insects in nature. Experimentally it is nonpathogenic in ordinary doses for wax-moth larvae, although large doses have been reported to be pathogenic for this insect. *Streptococcus faecalis* A. & H., a common inhabitant of the intestines of many animals, including some insects, is apparently pathogenic for the larva of the wax moth when injected in moderate doses.

MICROCOCCACEAE INFECTIONS

Of the three genera (*Micrococcus*, *Gaffkya*, and *Sarcina*) of the family Micrococcaceae, only the genus *Micrococcus* is of any real significance as far as insect pathogens are concerned. (Incidentally, the student should

bear in mind that, according to the latest (6th) edition of "Bergey's Manual," the members of the genus *Staphylococcus* are now included in the genus *Micrococcus*.) Of this group, many of the cocci have been reported from isolated cases of infection, and so we have numerous so-called "species" of micrococci with very little accompanying information with regard to the pathogenesis of the disease for which they are supposed to be responsible. In only one or two instances has the study been anywhere near thorough.

Micrococcus Infections

One of the better studied cases of micrococcus infection is that of June-beetle larvae (*Lachnosterna*), caused by *Micrococcus nigrofaciens* Northrup. Along with a prevalence of June-beetle larvae in 1912, Northrup (1914*a,b*) observed a considerable number of diseased specimens from which not only *M. nigrofaciens* but frequently a bacillus similar to *Bacillus septicus-insectorum* Krass. was isolated. The latter organism may, in fact, have been the primary invader in most instances of the disease, but the micrococcus was the more active and apparently caused the greatest damage to the host.

Micrococcus nigrofaciens presumably exists in the soil and is present in many soils in Michigan, Illinois, Maryland, North Carolina, and probably in most of the other states and countries. It grows well on ordinary bacteriological media but much better on media in which the triturated larva itself is incorporated. Storage of the organism on artificial media for periods of over a year does not decrease its virulence for the grubs.

Compared with a normal larva, the appearance of one infected with the micrococcus is characterized by the black and shiny aspect of the affected parts, which are sharply circumscribed. The joints of the legs, the spiracles, and the dorsal or ventral segments of the white soft portion of the body are the principal sites of infection. As the disease progresses the larva becomes almost entirely black or brownish in color until the entire body seems to be in a state of advanced putrefaction, yet the insect still shows signs of life. The predominance of the brownish color is thought to indicate the invasion, secondary or primary, by *Bacillus septicus-insectorum*. Younger larvae seem to be more susceptible than do older larvae. Within certain limits, neither the size nor the number of infected areas appears to affect the activity of the grub. Only under certain conditions (parasitic insects, fungi, mechanical injury, or other predisposing factors) will death occur prior to the time of pupation, when they may be unable to complete metamorphosis. Excessively wet soil favors the progress of the infection, and Northrup considers this factor as probably the most important one concerned in the fatality of the diseased grubs.

The progressive destructiveness of the infection may be best seen in the pathological changes that take place in the legs of the diseased insect. When the infection progresses up the leg, it turns black segment by segment and drops off, leaving the stumps shiny, black, and sometimes swollen in appearance. When the infection occurs at one of the intermediate joints, the leg breaks off at this point.

Histological sections of diseased portions of the larvae show the micrococci embedded in the laminae of the integument, and in the underlying hypodermal cells. Cocci are also found interspersed between the cells of the intestinal wall, but none is to be found in the tissues of the body cavity between this structure and the integument.

Insects other than *Lachnosterna* larvae are susceptible to *M. nigrofaciens*. These include the green June beetle, *Cotinis nitida* (Linn.), the May beetle, *Phyllophaga vandinei* (Smyth), and the rhinoceros beetle, *Strategus titanus* Fabr. Adult cockroaches (*Periplaneta americana* (Linn.)) are also very susceptible, although the infection is apparently limited to the legs of this insect. The eastern tent caterpillar, *Malacosoma americana* (Fabr.), has also been listed as being experimentally susceptible to the micrococcus. The use of *M. nigrofaciens* in the control of any of these insects in the field has not been investigated.

Other Micrococcus Infections. The first micrococci reported to have been recovered from diseased insects were apparently those isolated by Eckstein (1894) from larvae of the nun moth, *Lymantria monacha* Linn. He called them *Micrococcus major* and *Micrococcus vulgaris*. Both organisms were also experimentally pathogenic for certain other species of insects. In the years following these isolations a number of other species were described, several from lepidopterous hosts. For example, in 1926, Chittenden reported that in some seasons large numbers of cabbage-butterfly larvae, *Pieris rapae* (Linn.), are killed by a contagious bacterial disease caused by *Micrococcus pieridis* Bur. *Micrococcus saccatus* Mig. was found by Pospelov (1927) in dead larvae of *Euxoa segetum* Schiff. *Micrococcus curtissi*, described by Chorine (1929*a*), was observed to cause a high mortality among young larvae of the European corn borer, *Pyrausta nubilalis* (Hbn.). This micrococcus was also very virulent for full-grown borers when injected, and to a less extent when administered by mouth. By injection it is virulent to larvae of *Ephestia kühniella* Zell., but the larvae of *Galleria mellonella* (Linn.) were more resistant.

Larvae of the sawfly, *Neurotoma nemoralis* Linn., were observed by Paillot (1924) to suffer from a disease caused by *Micrococcus neurotomae* Paill. He did not find the coccus to be of any great help in checking the insect in nature. Experimentally, it is pathogenic to *Euxoa segetum* Schiff., and *Agrotis pronubana* Linn. The organism tends to stain gram-

negative. From diseased locusts a bacterium that he called *Micrococcus acridicida* was isolated by Kuffernath (1921). From houseflies, Glaser isolated *Micrococcus muscae* (Glaser) (*Staphylococcus muscae* Glaser). This coccus is responsible for a fatal infection in adult flies. The disease is rather sporadic and never assumes the form of an epizootic. Only about 50 per cent of the flies contract the infection when experimentally infected. Males appear to be more susceptible than females. Incidentally, this coccus is host to a bacteriophage, *Phagus liber* Holmes. *Micrococcus rushmorei* Brown is another micrococcus that has been found associated with diseased flies.

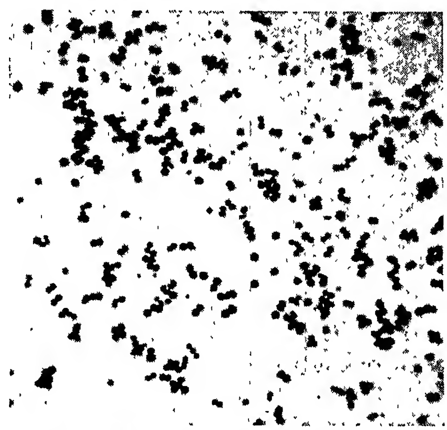


Fig. 93. *Micrococcus muscae* (Glaser) the cause of a fatal infection in adult houseflies. (*Courtesy of R. W. Glaser.*)

Two of the most common micrococci known, *Micrococcus pyogenes* var. *albus* (Rosen.) and *Micrococcus pyogenes* var. *aureus* (Rosen.) (formerly *Staphylococcus albus* Rosen. and *Staphylococcus aureus* Rosen.), frequently are found associated with normal insects, but they have also been reported in pathogenic relationships. *M. pyogenes* var. *albus* was found by Tauber (1940) in the hemolymph of the oriental roach, *Blatta orientalis* Linn., and was pathogenic to the roach. Just how the bacteria made their way into the hemolymph of the roach is not clear. He suggested that, after the insect molts, the exoskeleton is very soft and easily injured. Then the uninfected roach comes in contact with the infected ones, and the bacteria penetrate the delicate newly exposed exoskeleton or pass through breaks in the surface. *M. pyogenes* var. *aureus,* the cause of boils and other infections in man, has been found to be pathogenic for silkworm larvae. Although some workers have reported *M. pyogenes* var. *aureus* to be nonpathogenic for the larva of the wax moth, this insect does seem to be susceptible if adequate doses are used. Zernoff and Ajolo (1939) found the larvae to survive several lethal doses of the coccus if an injection of paraaminophenylsulfonamide is given simultaneously with the bacteria.

Among the gram-negative cocci (family Neisseriaceae), none have been recorded as true pathogens of insects. *Neisseria gonorrhoeae* Trev., the cause of gonorrhea in man, has been reported as both pathogenic and slightly pathogenic for wax-moth larvae, when injected into the body cavity of the insects. *Neisseria lucilliarum* Brown (probably a *Micro-*

coccus) has been recovered from green-bottle flies (*Lucilia sericata* Meig.) that had died of infection with *Flavobacterium lutzae* (Brown). What relation, if any, the small gram-negative encapsulated bacterium found by Glaser and Chapman (1912) in gypsy-moth larvae dying of a polyhedral virus disease has to the *Neisseria* group is not clear. Its discoverers named the organism *Gyrococcus flaccidifex* because of its "gyrating motility," but the validity of this genus has not been upheld. It is similarly questionable as to what credence should be given the variety of this organism designated by Brown (1927) as *Micrococcus flaccidifex danai* and considered by him to be the cause of a "wilt" disease of the larva of the monarch butterfly, *Danaus plexippus* (Linn.).

PSEUDOMONADACEAE AND MISCELLANEOUS BACTERIAL INFECTIONS

Each of the two tribes (Pseudomonadeae and Spirilleae) of the family Pseudomonadaceae has a few representatives that are pathogenic to insects under certain conditions. In no case, however, has the nature of the pathogenicity concerned been well investigated.

Pseudomonas aeruginosa (Schroeter) (= *Bacillus pyocyaneus* Gess.), a common bacterium which produces a green water-soluble pigment and which occasionally is found in wounds, has been found experimentally to be pathogenic for several species of insects. For example, larvae of the wax moth, *Galleria mellonella* (Linn.), are very susceptible to even small doses of this bacterium when inoculated into the body cavity of the insect. The same fact has been reported in the case of the silkworm. A laboratory epizootic among *Schistocerca gregaria* Forsk. caused by *P. aeruginosa* has been reported by Lepesme (1937*a*), who also found it to be a secondary invader to *Aspergillus flavus* Link in this grasshopper.

Another bacterium of this group, *Pseudomonas septica* Bergey *et al.*, was believed by Stutzer and Wsorow (1927) to be one of the causes of a "spring disease" among caterpillars of *Euxoa segetum* (Schiff.). They were able to reproduce the disease experimentally by infecting the insects through the damaged integument.

Of the tribe Spirilleae, *Vibrio leonardii* M. & C. (= *V. leonardi*) appears to have been the first entomogenous species isolated. Metalnikov and Chorine (1928*a*) recovered this organism from diseased larvae of the European corn borer and found it to be very pathogenic for this insect and for wax-moth larvae. The insects were very susceptible by mouth, dying in 24 hours. Another species, *Vibrio pieris* Paill., is referred to by Paillot (1933) as having been frequently encountered in caterpillars of *Pieris brassicae* (Linn.) that were parasitized by larvae of *Apanteles glomeratus* (Linn.).

Vibrio comma (Schroeter), the cause of cholera in man, is pathogenic

for wax-moth larvae when these insects are inoculated with the bacteria directly. It is also associated with such insects as flies and cockroaches which serve as mechanical vectors of the organism. Although not belonging to the same group, another human pathogen might be mentioned here because of its deleterious effect upon its insect host. *Pasteurella pestis* (L. & N.) (family Parvobacteriaceae), the cause of plague in man and rodents, multiplies rapidly in the proventriculus of its flea vector until it "blocks" the flea, causing it to regurgitate when it again attempts to feed. Blocked fleas do not live so long as do normal fleas. *Pasteurella tularensis* (McC. & Chapin), the cause of tularemia in man and other animals, invades the gut epithelium and coelomic cavity of its tick host, although this usually does not constitute a disease of the arthropod as such. However, infected ticks may die earlier than uninfected ticks.

The organism *Leptothrix buccalis* (Robin) (= *Leptotrichia buccalis* (Robin)) was found apparently infecting *Anopheles maculipennis* Meig. by Perroncito in 1899. According to Keilin (1921), "The parasite infests the larva, passes into the pupa, and destroys the imago soon after it emerges."

SPIROCHAETALES INFECTIONS

The order Spirochaetales is comprised of two families. The first (Spirochaetaceae) contains three genera: *Spirochaeta, Saprospira,* and *Cristospira.* The second (Treponemataceae) contains *Borrelia, Treponema,* and *Leptospira.* Members of the family Spirochaetaceae are larger in size and less susceptible to the action of bile salts than are Treponemataceae. One outstanding difference between spirochetes and true bacteria is that the latter have a rigid cell wall while the spirochetes are flexible.

Compared with the true bacteria and true protozoa, the number of spirochetes known to be associated with insects and ticks is not large. Nevertheless, many of those instances in which such associations do occur are very important from the standpoint of health and economics. Most important in this regard is the relapsing-fever group of spirochetes. In addition some entomogenous spirochetes are nonpathogenic for man and animals, and some are found in only a fortuitous association with arthropods.

The relapsing-fever group of spirochetes (*Borrelia recurrentis* (Lebert), etc.) is transmitted by lice (*Pediculus humanus* Linn.) and by ticks (*Ornithodoros* spp.). The spirochetes do not cause a disease of the arthropods as such, but they do invade the tissues of the arthropods which may then, in a sense, be considered infected. Some ticks become immune to this type of infection by the spirochetes. Similar relationships exist between *Borrelia anserina* (Sakh.) (the cause of a septicemia in chickens) and its

vector, *Argas persicus* Oken. *Borrelia theileri* (Lav.) is the cause of an infection in cattle and is transmitted by ticks and passes through the egg of these vectors.

The tissues of some insects have been found to contain spirochetes but whether or not this represents a true infection is not known. For example, *Spirochaeta culicis* Jaffé has been found in large numbers in the salivary glands of *Anoheples maculipennis* Meig., as well as in the intestinal tracts and Malpighian tubes of other mosquitoes (*Culex*). Furthermore, a considerable number of spirochetes have been found in insects, particularly in their alimentary tracts, of which the relationships to their hosts are not definitely known. For the most part these may be harmless commensals, although some, like *Spirochaeta ctenocephali* Patton in the cat flea (*Ctenocephalides felis* (Bouché)), may be parasitic.

Spirochete Septicemia in Caterpillars. In 1940 Paillot described a septicemia in caterpillars of the cabbage butterfly, *Pieris brassicae* (Linn.), and named the causative organism *Spirochaeta pieridis*. The blood of larvae infected with this spirochete has essentially the same appearance as is exhibited in the case of a bacterial septicemia. There are, however, no external symptoms that distinguish sick caterpillars from healthy ones.

The spirochetes are found principally in the blood, although some hypodermal cells are affected and spirochetes may occasionally be seen in the intercellular spaces of the fat tissue. Phagocytes take up a considerable number of them. Frequently, a secondary bacterial septicemia is also present.

The infection cannot be initiated by the digestive route but can be through the skin, although even this does not always succeed. Experimentally, the silkworm (*Bombyx mori* (Linn.)) is not susceptible to *Spirochaeta pieridis*.

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CHAPTER 10

FUNGOUS INFECTIONS
(Mycoses)

As far as the diseases of insects are concerned, it seems only natural that the first type of infection definitely identified as to its microbial nature was that caused by fungi. This relationship was suspected about the beginning of the nineteenth century (see Kirby and Spence, 1826). The conspicuous presence of a mycelium and fruiting bodies on the cadavers of dead silkworms enabled Bassi de Lodi, in 1835, to recognize the similarity between the infecting fungus and the well-known bread molds and other saprophytic fungi. As further observations were made it was discovered that some of the fungi infecting insects were obligate parasites, others were semiparasites, and still others were common saprophytic species which, under certain conditions, were able to cause a frank infection in susceptible insects. A confusing factor was the frequent development of a saprophytic fungus in the body of an insect killed by an agent other than the fungus. Such secondary invaders were often mistaken for the primary cause of the condition, and as a result the invasive properties of some of the fungi described in early reports are in doubt and these organisms probably are not pathogenic agents in the true sense of the term.

In an earlier chapter we referred to certain fungus-insect relationships that could not properly be considered in the category of disease (*e.g.*, the *Septobasidium* fungi and the Laboulbeniaceae), and others that are of a definitely mutualistic association (*e.g.*, the ambrosia fungi of termites and wood-boring beetles). These relationships are therefore outside the limits of the usual concept as to what constitutes a fungous disease.

Nature of the Fungi Concerned. Fungi constitute a part of that subdivision of the plant kingdom known as Thallophytes, those organisms which grow in irregular plant masses (thalli) not differentiated into roots, stems, and leaves as in higher plants. The term "fungi" is a general one and may be meant to include several types of organisms that are sometimes conveniently differentiated by the terms "molds," "yeasts," "actinomycetes," "bacteria," etc. Commonly, however, the term "fungi" refers to that group of nonchlorophyll-containing organisms which usually possess the filamentous vegetative structure known as "mycelium." Or, stating it another way, the word "fungi" usually refers specifically to the molds and yeasts, which comprise the Eumycetes.

Representatives of the fungi known to cause infection and disease in insects ("*fungi Entomogeni*") are included in each of the four classes: Phycomycetes, Ascomycetes, Basidiomycetes, and Deuteromycetes (Fungi Imperfecti). Of these, the class Basidiomycetes contains the fewest species characteristically pathogenic for insects. Because, under certain conditions, some groups of yeasts produce asci and ascospores, these microorganisms are usually included with the Ascomycetes.

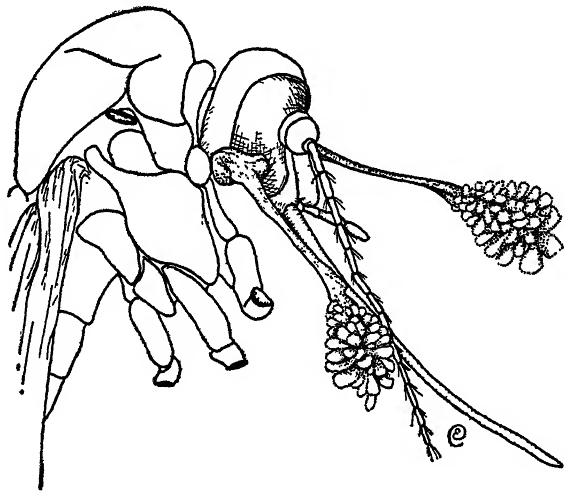


Fig. 94. The anterior portion of a mosquito bearing pollinia, which are frequently mistaken for fungi. Such artifacts usually become detached if they are moistened with a drop of alcohol. (*From a specimen collected by C. R. Twinn.*)

For the sake of convenience we shall treat the mycoses of insects more or less according to the class to which the causative fungus belongs. There is some overlapping between the fungi belonging to the class Ascomycetes and those placed in the class Deuteromycetes. The latter group are sometimes called "Fungi Imperfecti" because they are fungi with incomplete or incompletely known life cycles. In most cases they are believed to be imperfect (asexual) stages of Ascomycetes. The details of these relationships will be elaborated upon in a later section.

In passing, mention should be made of the fact that certain artifacts, foreign bodies, or pseudofungi occasionally occur on insects and are sometimes confusing to one examining the specimens for fungi. Certain seeds and pollinia, for example, frequently are found attached to the integuments of insects in a manner closely resembling certain fungi such as Laboulbeniaceae and *Cordyceps*. Usually these foreign nonparasitic bodies may be detached mechanically from the insect rather easily, although sometimes they cling as tenaciously as might be expected of a fungus.

Pollinia may be attached by adhesive discs, which will ordinarily become detached from the insect if the specimen is moistened with a drop of alcohol. Some orchid pollinia, with their stalks of varying lengths and pollen masses at the apex, are similar in appearance to some of the stalked fungi but become detached readily upon the application of alcohol. The pollen of some plants covers insects (such as wasps) visiting their flowers with a white powder that is sometimes mistaken for a fungus, but this can usually be detected upon microscopic examination.

PHYCOMYCETE INFECTIONS

Phycomycetes ("algal fungi") constitute a large and diverse group living in various habitats; some are aquatic, some amphibious, some terrestrial. They are characterized by coenocytic (nonseptate and multinucleate) mycelium and endogenous asexual spores. Septations commonly are formed where the reproductive structures are delimited; they may be formed in old hyphae of certain genera and in young thalli of certain other genera. From the standpoint of insect infections, at least four orders of the class Phycomycetes contain entomogenous members: Entomophthorales, Mucorales, Blastocladiales, and Chytridiales. As the name would imply, the most important of these is the order Entomophthorales, which is generally considered to consist of a single family, Entomophthoraceae (Empusaceae of some authors), which in turn may be divided into five and possibly six genera. Of these, the genera *Empusa*, *Entomophthora*, and *Massospora* are composed primarily of entomogenous species.

Infections Caused by Species of *Empusa* and *Entomophthora*

The literature records a relatively large number of infections of insects caused by species of *Empusa* and *Entomophthora*. Approximately 50 species of these fungi are known to occur in the United States. Some of these have been studied in considerable detail whereas others have received only taxonomic descriptions. In the main, their life histories are similar, and considerable general information may be gained by a close consideration of a few of the better known species.

The proper use of the generic names *Empusa* and *Entomophthora* is frequently a difficult procedure, and unless the student has an understanding of the historical aspect of these names he is likely to become somewhat confused. No final official ruling has been made on this particular nomenclatorial problem, and for the present one can only follow the usage generally employed by the leading authorities in the field. The validity of the name *Empusa*, erected by Cohn in 1855 for a fungous parasite (*Empusa muscae* Cohn) of the housefly, has been challenged by some writers because of its having been preoccupied for a genus of orchids.

It was for this reason that Fresenius (1856) proposed the name *Entomophthora* to take the place of *Empusa* for the fungus. This was followed by the indiscriminate use of both names until Brefeld (1877) and Nowakowski (1884) separated them as two distinct genera, thereby recognizing the validity of the name *Empusa*. Other mycologists subsequently employed it without question. In his treatise on the Entomophthoreae of the United States, Thaxter (1888) concluded that the name had suf-

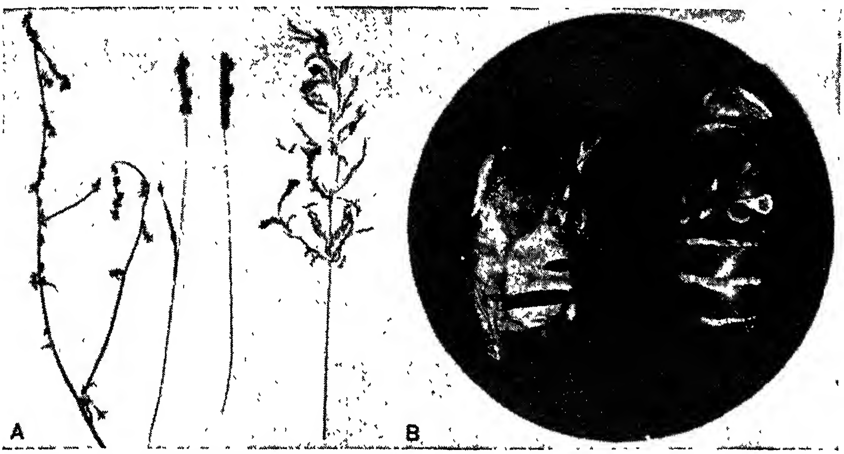


Fig. 95. Blowfly, *Phaenicia mexicana* (Macq.) (= *Lucilia unicolor* Town.), killed by an entomophthorous infection (*Empusa americana* Thaxter). *A*. Blowflies clinging to the stalks of chicory and grasses. Collected from a rather extensive epizootic in the field. *B*. Close-up of specimens killed by the fungus. Note tendency of conidiophores to break out of the body cavity along intersegmental membranes. (*Courtesy of E. Dresner, Boyce Thompson Institute, and Ohio State University.*)

ficient weight of authority to make it acceptable. He further pointed out that the orchidaceous genus *Empusa* is now placed as a synonym and hence seems unlikely to cause confusion.

The generic name *Entomophthora* was considered by Thaxter to be of subgeneric value in the genus *Empusa*. Since then, for reasons to be pointed out later, there has been a tendency to revert to the proposals of Brefeld and Nowakowski and to give *Entomophthora* full generic rank along with *Empusa*. The genus *Lamia*, erected by Nowakowski and considered by him to be intermediate between the two, has not found general acceptance. Unfortunately, a clear and final distinction between some of the species of *Empusa* and those of *Entomophthora* has not been made. It appears probable, however, that the two names will be used separately and that with further detailed studies two valid genera will be recognized.

Nature of *Empusa* and *Entomophthora* Infections. Among the various species of *Empusa* and *Entomophthora* there is some variation as to the

details of the manner in which they cause infection in their respective hosts, but in general this process is much the same throughout the two genera.

Infection of the insect by the fungi via the alimentary tract probably occurs only rarely, if at all. The usual route of infection is by the penetration of the integument, especially the thinner intersegmental areas of the body wall and the appendages. Soon after the fungous spore comes in contact with the integument it begins to germinate, sending out a conidial hypha that penetrates into the body cavity of the host where it develops rapidly at the expense of the softer tissues. Instead of producing a profusely branched mycelium, the hyphae usually become segmented and break down into their component cells, which are called "hyphal bodies." These short thick fragments of varied size and shape, containing a highly concentrated fatty protoplasm, multiply by budding or by fission until the insect's body cavity is filled with them. In some cases this fragmentation of the hyphae does not occur until later in the infection, after the mycelium is fairly well developed. The hyphal bodies may be very irregular in size and shape, or they may possess great regularity in this regard. The latter is frequently the case with hyphal bodies produced about the time of the host's death and just preceding spore formation.

After the hyphal bodies have been produced, if conditions of temperature and moisture are not favorable, instead of completing its development, the fungus may form chlamydospores. These spores are formed by each hyphal body which develops about itself a single wall of variable thickness depending upon the duration of this resting stage. By means of the chlamydospores the vitality of the fungus may be maintained through long periods of dormancy until the proper conditions for further growth are presented. The period from first infection to the formation of hyphal bodies or chlamydospores varies according to the host from 2 to 12 days. In general, the smaller the insect the shorter the period.

In the presence of sufficient moisture and adequately high temperature, the hyphal bodies and chlamydospores "germinate" with great rapidity. The hyphae thus produced may grow directly to the outer air and then produce a single conidium or set of conidia, according to the type of conidiophores. Sometimes, particularly when conditions of growth have been very favorable, a single primary hypha may branch indefinitely, each ultimate branch becoming a conidiophore. As a rule, the number of germinating hyphae that develop from a single hyphal body does not exceed one or two, although instances occur in which the number is considerable. In the latter case the hyphae may branch and anastomose, forming a coherent mass known as a "stroma."

As a result of the germination of the hyphal bodies either sexual or

asexual resting spores (zygospores or azygospores), or conidiophores bearing conidia are produced. In the case of the latter, as explained by

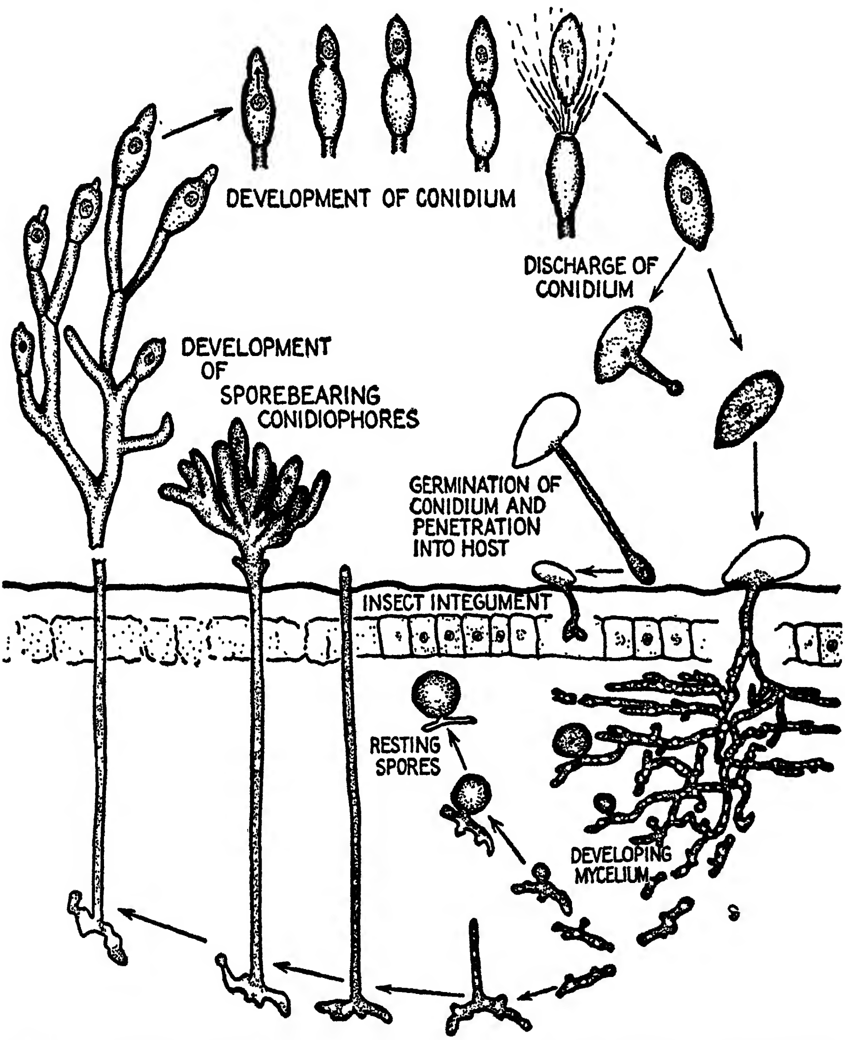


Fig. 96. The principal stages of the developmental cycle of an entomophthorous fungus, *Entomophthora sphaerosperma* Fres. The lower half of the figure represents the type of development that takes place within the body of the insect; the upper half represents that which occurs on the surface or outside the insect.

Thaxter, the hyphae, arising directly or indirectly from the hyphal bodies, grow rapidly outward and burst through the less resistant portions of the host's integument in spongy masses. In most instances the color of these

masses is white, although the hues may vary from bluish white to pale or bright green or dull olive. There may be considerable variation in their general appearance, depending upon the species of fungus or the conditions of their development. In some cases the masses project barely beyond the body wall of the host and are confined to the points of emergence. These points are usually the thin intersegmental membranes through which the fungus projects in cushionlike rings, usually formed by simple conidiophores producing few or no branches outside the host's body. Each of these conidiophores gives rise to a single conidium. In other cases the external growth may be more extended, and the masses may coalesce so as to cover the entire body with a continuous layer of conidiophores which may form a mass several times as large as the insect itself. The external parts of these conidiophores are considerably branched, and the ultimate divisions of each conidiophore are arranged in a corymbose or digitate fashion. It is this occurrence of simple and compound conidiophores in different species which has led to the establishment of the two genera *Empusa* and *Entomophthora.* The difficulty in maintaining this generic separation is that the distinction is not absolute, and intermediate forms occur (*e.g.*, in *E. culicis* Bra. and *E. apiculatus* Thax.). Compound conidiophores are sometimes found in species usually having the simple type, and simple conidiophores are commonly found in species having the compound type. In either case the growth of the conidiophores under optimum conditions of temperature and moisture takes place very rapidly and may give rise to the characteristic white masses in a few hours. Soon after the masses of conidiophores appear, the production of conidia begins.

Thaxter explains the formation, discharge, and germination of the conidia as follows: The terminal portion of the conidiophore is termed the "basidium" and is usually swollen to a greater or less extent. From the apex of this basidium, the conidium commences its formation by the process of budding. The bud, or mother cell, increases until it reaches the normal size and shape of the conidium; then it becomes separated from the basidium by a cross partition. Within the mother cell thus formed is developed a single conidial spore. When the conidium is fully developed, the contents of the spore, as well as that of the basidium, begin to expand through the absorption of water. At first the contents of the basidium exert the greater of the two forces thus produced, and the columella is forced outward into the conidium toward which its convexity is thus turned. When the basidia are large and strong, this process may continue until the discharge of the conidium. Usually the contents of the conidium, being more dense than that of the basidium, finally exert a greater pressure and force the columella back into the basidium, thus reversing its former

position. The sum of these opposing forces is very great, and as a result of their action a rupture of the wall takes place at the point where they are opposed—in a circle around the base of the mother cell. As a result of this rupture, the conidium is discharged violently into the air, often to a considerable distance. The columella commonly remains unbroken by this discharge, although it may be greatly stretched and hang down from

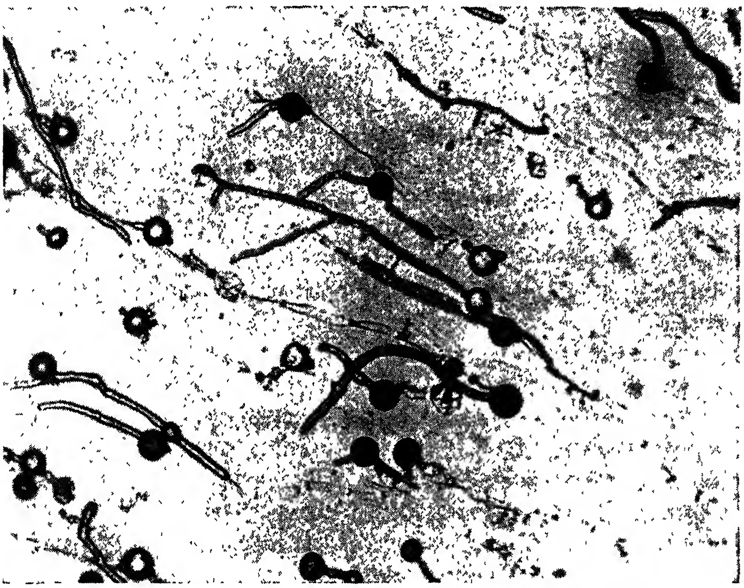


Fig. 97. Germinating conidia of *Entomophthora coronata* (Cost.), a parasite of termites, aphids, and probably other insects, showing papillae, secondary conidia, and segments of mycelium both empty and filled with protoplasm. (*From Harris, 1948.*)

the basidium as a tonguelike projection. In some instances, however, it may be broken or remain connected to the discharged conidium.

The conidia of the different species of the tribe Entomophthoreae vary in size and form; in fact, marked variation is sometimes apparent in the conidia of the same species. In size they range from about 10 to 75 microns in length. With regard to shape, the conidia vary from being spherical to being slender and tapering. They are usually hyaline, rarely slightly colored, and contain fine granular protoplasm or, more commonly, coarsely granular protoplasm with large fat globules. These fat globules may be so regular in size and shape that the conidia resemble asci filled with spores. The walls of the conidium are smooth, without spines or similar modifications, and possess an adhesive quality that serves to attach them to any object with which they come in contact. The basal portion of the spore is more or less papillate.

When discharged, the conidium, if it comes in contact with a suitable host, adheres to it and sends out a germination hypha, which enters its body in the manner already described. If the conidium lands in water, it gives rise to one or more hyphae that branch and elongate, growing constantly more attenuated, their protoplasmic contents becoming separated by successive cross partitions from the empty hyphae left behind. This growth may continue until the protoplasm is spent. The separation by cross partitions is common in the general growth of the fungus.

The process of germination is extremely variable but rarely lasts longer than a week and is usually much shorter than this. Germination ordinarily takes place soon after the conidium is discharged. If the discharged conidium falls neither on a proper host nor upon a wet surface, it proceeds to form what are called "secondary conidia," a process that provides for further dissemination if the primary spore has fallen upon a substance unsuited for its proper development. The most common method of formation, according to Thaxter, consists in the production of a hypha of variable length which, growing vertically upward, becomes swollen at its extremity into a basidium and produces a conidium usually similar to that from which it originated. This secondary conidium is discharged in the usual fashion. It, in turn, may produce tertiary conidia, and the process continues until its vitality is exhausted or until it has come in contact with a suitable host. With some species, under unfavorable moisture conditions, the appearance of the secondary conidia may differ considerably from that of the primary conidium. They are usually almond-shaped, have thick walls, and are not discharged.

Occasionally, upon the examination of a conidiophore mass, a simple hypha is seen exceeding the conidiophores in size and projecting beyond them, often to a considerable distance. Some of these hyphae, called "cystidia," are so large that they may be readily seen with the naked eye; others are not much different from the ordinary conidiophores. The function of the cystidia is not known unless, as Thaxter (1888) suggests, they are rhizoids or hyphae of attachment but functionless because of their position. The functioning rhizoids are hyphae that attach themselves to the substratum upon which the host rests and serve to hold the fungus firmly in position. The rhizoids may be simple or branched, and their termination may be modified into an expanded suckerlike structure of attachment. Rhizoids appear to be confined to certain species and usually accompany the digitate type of conidiophores.

Formation of Resting Spores. Within the body of the insect infected with an Entomophthoreae, a process or phenomenon frequently occurs by which special spores are formed that are highly resistant to conditions

that would ordinarily destroy the conidia. These "resting spores," as they are called, may be formed by an asexual process, in which case they are known as "azygospores," or they may be formed by sexual union and then are known as "zygospores." The resting spores are usually spherical, of large size, contain highly refractive fatty contents, and are surrounded by triple walls. The outer wall of the spore is thin and represents the wall of the mother cell, the second is thicker, and the innermost wall is usually as thick as or thicker than the other two combined.

Azygospores may be formed in a variety of ways. The simplest process is that by which the contents of a hyphal body become directly converted into a resting spore. Or azygospores may be formed from hyphae of germination arising from chalmydospores or hyphal bodies, or by direct lateral budding from them. Sometimes azygospores may be produced interstitially—between fungous cells—and this frequently results in spores having very irregular shapes.

Zygospores are also formed in a variety of ways, although sometimes the sexual nature of the spore is not well marked, in which case this may represent a transitional form from the truly sexual to the entirely asexual processes. The method of true zygospore formation is, as might be expected, by conjugation of two different hyphal outgrowths; these meet, the intervening walls are absorbed, and the contents of the two mingle. In many cases a bud then appears on one or both of the gametes, increases rapidly, and becomes the zygospore. Sometimes the spore develops as a terminal swelling from the end of one of the conjugating hyphae. Other methods of formation have been observed.

Hosts and Habitats of Entomogenous Entomophthoraceae. Any attempt to summarize the insect hosts of a particular group of microorganisms must take into account the fact that the host distribution is to some extent dependent upon the relative amount of study made of the groups concerned. In the case of the insect hosts of Entomophthoraceae, the Diptera appear to be the order of insects the members of which are found to be the most frequently infected, and from which the greatest number of species of this group of fungi have been isolated. The Hemiptera are the next greatest sufferers, followed by the Lepidoptera and Coleoptera. Certain species of Orthoptera, Hymenoptera, and Neuroptera are also known to be susceptible to these fungi. The adult stage of the host insect is affected more frequently than either the larvae or pupal stage. In insects with incomplete metamorphosis the nymph is almost as susceptible as the adult.

Although to a degree there is some specificity of hosts for each species of the tribe Entomophthoreae, this is by no means certain or uniform. Some species of Entomophthoreae infect a wide range of hosts, including insects

in different orders; others have been found only on a single insect species or on a closely related group of insects. Not infrequently two different species of fungi are found on one species of host or even on a single host.

Entomogenous Entomophthoraceae are found in a variety of habitats, but usually they develop best in areas where there is a constant and rather abundant supply of moisture. The edges of ponds and brooks in shaded places are a particularly suitable environment for many of them. Some species do well in drier situations as long as periods of occasional moisture occur, enabling conidia to be produced. Damp foggy weather frequently makes the fungi more conspicuous, since the moisture causes them to become distended.

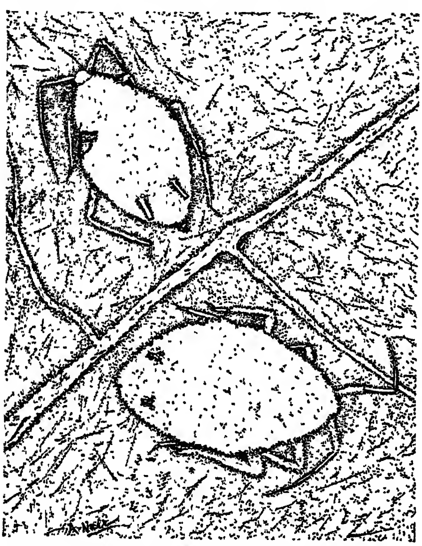


Fig. 98. Aphids (*Myzus persicae* (Sulzer)) killed by *Empusa aphidis* Hoff. and covered with the fungus.

Just before dying from the infection, many hosts seek elevated positions, crawling upward on blades of grass or other food plants. This, incidentally, makes it possible for the conidia to be discharged over a considerable area. Other favorite positions assumed by the infected insects are on the undersides of leaves in shady areas or about houses.

Cultivation of Entomogenous Entomophthoraceae. Only a few species of entomogenous Entomophthoraceae have been cultivated apart from their insect hosts. The fact that success in this regard has been obtained with a few species indicates that probably most of the remaining species can be cultivated if the right combination of substratum and growing conditions can be found.

The first to obtain the complete life cycle of one of these fungi in artificial culture apparently was Speare (1912), who succeeded in thus cultivating *Entomophthora pseudococci* Speare, a parasite of mealybugs in Hawaii. As media he used potato, potato agar, oat agar, and radish, the latter two being found the most suitable. In 1929, Sawyer reported the cultivation of *Entomophthora sphaerosperma* Fres. and an unidentified species of *Empusa*, the most satisfactory media in this instance being potato, swordfish, and pork.

Sawyer's studies revealed several interesting features connected with the cultivation of the two species of entomophthoraceous fungi with which he worked. For one thing, liquid nutrient media favored luxuriant mycelial growth, whereas solid media favored the production of hyphal bodies and reproductive phases. Carbohydrates and fats did not appear to be essential to the growth of these fungi, but the substratum had to contain proteins that are quickly liquefied by the proteolytic enzymes secreted by the fungi. The hydrogen-ion concentration of the media was also important, with pH values slightly on the acid side of neutrality being the optimum for growth and development of the organism. Atmospheric humidity did not materially influence growth or reproduction, although too much moisture in the substratum inhibited the latter. Conidia did not germinate below a relative humidity of about 70 per cent at 21°C. This temperature (21°C.) appeared to be the most favorable for growth and reproduction; the maximum temperature was 34°C. The exact minimum was not determined, but Sawyer found that the conidia of both species with which he worked could be frozen for several days and still germinate upon return to room temperature. Vegetative growth and the production and germination of conidia took place in total darkness as well as in light. Best growth was obtained when large quantities of inoculum were transferred to the media used. Cultures held at room temperature were best transferred once every 10 days.

The artificial propagation of entomogenous Entomophthoraceae may also be accomplished by infecting fresh hosts. Thaxter (1888) developed a method of doing this by using a tightly covered jelly tumbler in which the upper portion is separated from the lower by a round piece of wire netting. By placing the hosts to be infected in the lower of the two chambers and fastening a specimen carrying the fungus in the upper one, Thaxter found that the living hosts below acquired the discharged spores through the netting and thus became infected.

Examples of *Empusa* and *Entomophthora* Infections

It is not practical for us to attempt here a discussion of all the species of entomogenous Entomophthoraceae and the infections they cause. The essential relationships involved may be exemplified, however, by a brief consideration of a few of the better known species.

Since the systematics of this group of fungi is still unsettled, we shall not be too concerned about the eventual validity of the generic names to be used in certain cases. Inasmuch as most modern mycologists use the name *Empusa* to include species of Entomophthoreae having simple or unbranched conidiophores and multinucleate conidia, and the name *Entomophthora* for species with digitate or branched conidiophores and

uninucleate conidia, we shall do the same. Nowakowski's genus *Lamia* is not now generally recognized, and we shall not use it here.

***Empusa muscae* Cohn.** The first description of this fungus, commonly found infecting the housefly, *Musca domestica* Linn., was that given by DeGeer in 1782. About a century ago, Cohn (1855) gave it the name

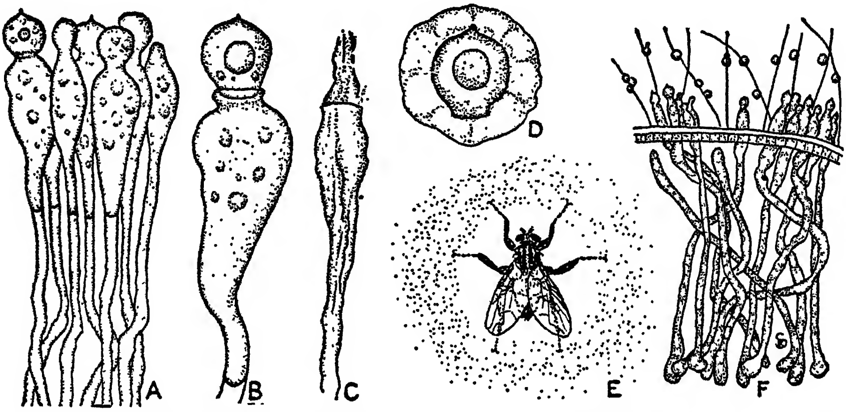


Fig. 99. *Empusa muscae* Cohn, the fungus commonly found infecting the housefly and other flies. *A*. A group of conidiophores showing conidia in several stages of development. *B*. Basidium bearing conidium before discharge. *C*. Basidium after discharge of conidium. *D*. Conidial spore discharged upon a glass slide and surrounded by a mass of protoplasm from the basidium. *E*. A fly infected with *E. muscae*, the ejected spores forming an aureole about the dead insect. *F*. Mycelium and conidiophores penetrating cuticle of insect (diagrammatic). (*A–D redrawn from Thaxter, 1888; E and F redrawn from Paillot, 1933, after Brefeld.*)

Empusa muscae, and it stands today as the type species of the genus. A considerable number of Diptera, in addition to the housefly, are subject to attack by this fungus, the most common of which are reported to be species of the genera *Lucilia* and *Calliphora*. Syrphidae are also susceptible. Distribution is apparently world-wide; most of the significant observations on the fungus have been made in the United States, Europe, and South America.

Infected flies are usually found indoors; they attach themselves to the walls and ceilings of houses and other buildings in the lifelike position. Close inspection of flies killed with the fungus usually reveals on the wall or windowpane a distinct halo of discharged spores encircling the insect.

The life cycle of *Empusa muscae* in most respects is essentially of the general type already described in the preceding pages. The species may be distinguished by its relatively large pointed bell-shaped spores each of which usually contains a single large oil globule. The pellicle of protoplasm (from the basidium) that surrounds the spores when discharged

on such a surface as that of a glass slide gives them a characteristic appearance (Fig. 99*D*).

Empusa grylli **(Fres.).** Fresenius (1856, 1858) originally described this fungus from a ground beetle under the name *Entomophthora Grylli*; but since the conidiophores are of the simple type, the generic name *Empusa* is probably more applicable, and it has been recognized as such by most subsequent taxonomists. The conidia of this fungus are ovoid to pear-shaped, the conidiophores may coalesce externally when growing luxuriantly, cystidia are wanting, and the resting spores are spherical and colorless. Its hosts include numerous species of Lepidoptera and Orthoptera, especially short-horned grasshoppers. Its prevalence on grasshoppers or locusts accounts for the designation "common locust fungus," often applied to it.

One of the first reports in American literature having reference to the destruction of locusts by a fungous infection is that of Bruner (1883) in which the author states that numerous instances of "internal fungoid growths" had come to his attention during the preceding 12 years. In 1896, in South Africa, grasshoppers had been found dying from a fungous disease. Some of the diseased specimens were sent to the United States, and a fungus was isolated. This unidentified fungus was distributed to planters, who in many cases reported considerable destruction of grasshoppers by the use of the microorganism. The possibilities attending the use of fungi in the control of grasshoppers appeared promising enough to initiate further studies, which were promoted under the direction of L. O. Howard of the Bureau of Entomology of the U.S.D.A. In 1902 Howard summarized the various reports on the effectiveness of fungi as a control agent and concluded that the results obtained did "not justify very sanguine hopes." He also pointed out that some of the cultures being distributed by various agencies and laboratories were not *Empusa grylli*. Some of them were saprophytic contaminants, and others were species of *Mucor* that did seem to have some insect-killing properties. A record was also made of the susceptibility of grasshoppers to the so-called "chinch-bug fungus," *Beauveria globulifera* (Speg.). In the years following Howard's report, the artificial use of *Empusa grylli*, as well as other fungi, as an agent of insect control, lost favor. Its destructiveness in natural outbreaks was recognized, but its effectiveness was too dependent upon optimum conditions of temperature and moisture to be a practical means of artificial control.

The symptomatology of an *Empusa grylli* infection in grasshoppers is fairly characteristic. Skaife (1925), in South Africa, has described the disease somewhat as follows: The dying insects climb as high as they can on the grass stems and on the twigs of bushes with their heads pointing

upward. Just before death, which usually occurs about 5 or 6 days after infection takes place, they loosen their hold with their claws and embrace the stems with their legs. After death, which occurs while they are in these elevated positions, their legs stiffen and the dead bodies remain hanging in this position for several days until they are finally blown away by the wind or washed down by the rain. Skaife observed that the majority of the insects infected with the fungus die in the late afternoon, usually between 3 and 7 P.M. An hour or so after death a fine furry or velvety growth appears growing from the intersegmental membranes, from the joints of the legs, around the neck, and at the base of the antennae. This growth usually has a white, buff, or greenish color, and it consists of innumerable club-shaped conidiophores that project from the insect's integument. At the end of each conidiophore a conidium is produced. These conidia are discharged generally in the evening when the live grasshoppers are clustered together for the night. This, of course, facilitates the transmission of the fungus from the diseased insect to a healthy one. According to Skaife, there are several different strains of *Empusa grylli* varying in virulence, and apparently individual grasshoppers vary in their susceptibility to any particular strain. About 1 per cent of the dead grasshoppers fail to produce the external growth of conidiophores. When these insects are opened they are usually found to be filled with the thick-walled, resistant resting spores.

In South Africa, Skaife found that the disease develops only in those localities which had a rainfall of over 14.5 inches during the 6-month period. The disease did not appear in four areas that had received over 4 inches of rain in only 1 month. A rainfall of 14 to 15 inches over a period of 3 months, however, appears to be sufficient to start the disease in South Africa. In the United States it is generally agreed that warm humid or wet weather is necessary to enable the infection to develop. During dry weather there is practically no extension of the disease. It is unfortunate that grasshoppers do their greatest damage in the dry seasons, when conditions least favorable for the development of the fungus prevail.

Entomophthora sphaerosperma **Fres.** One of the best studied entomophthorous fungi is *Entomophthora sphaerosperma*, a fungus first recorded as a parasite of the cabbage butterfly of Europe, *Pieris brassicae* (Linn.), by Fresenius (1856, 1858), who briefly described it and gave it its present name. The fungus has several times been known by other names (*Tarichium sphaerosperma* Cohn, *Empusa radicans* Brefeld, *Entomophthora radicans* Brefeld, *Entomophthora phytonomi* Arthur, and *Empusa sphaerosperma* (Thaxter)), but the name and combination originally used by Fresenius have retained their validity. It was first reported in the United States by Arthur (1886)—under the name *Entomophthora phyto-*

nomi—as a parasite of the clover leaf weevil, *Hypera punctata* (Fabr.). Since then it has been found on a wide variety of hosts in many parts of the world. In fact, its host range includes nearly every order of insects, the most common being Diptera, Hemiptera, and Lepidoptera. In numerous instances insects are subject to widespread outbreaks of infection by this fungus, which thus is frequently of considerable economic importance in the natural control of certain insect pests.

The morphology and development of *Entomophthora sphaerosperma* has been described by several authors, but one of the most complete accounts is that by Sawyer (1931). This worker succeeded in growing the fungus on swordfish and potato. From such cultures, which grew best at temperatures of from 18 to 21°C., Sawyer was able to make a careful comparison of the various stages in the life cycle of the fungus. The conidia are narrowly elliptical, with a rounded apex and a tapering base, and have an average size of 7 by 22 microns. A single membrane encloses the spore, and the apex is frequently covered by a detachable, transparent gelatinous cap. The protoplasm is finely granular and nonvacuolate in the newly formed spore, although vacuoles begin to form as soon as the spore has a single centrally located nucleus. The mature conidiophores are digitately branched at the distal end. A conidium develops at the end of each branch, a thin wall separating it from the conidiophore, which has also formed a cross wall at this point. After a given interval the spore is violently discharged. If the spore lands in a suitable environment germination begins with a slight outward bulging of the conidium at any point on its surface. As growth proceeds, a tube is formed at the tip of which may arise a secondary conidium; or, if it is able to penetrate a susceptible host, the germ tubes may continue to develop, forming a mycelium and hyphal bodies. Rhizoids may also eventually be formed. These structures usually arise from the hyphae in the thoracic region of the insect and attach the host firmly to the substratum. The rhizoids constitute the only structure of the fungus that is not developed on artificial

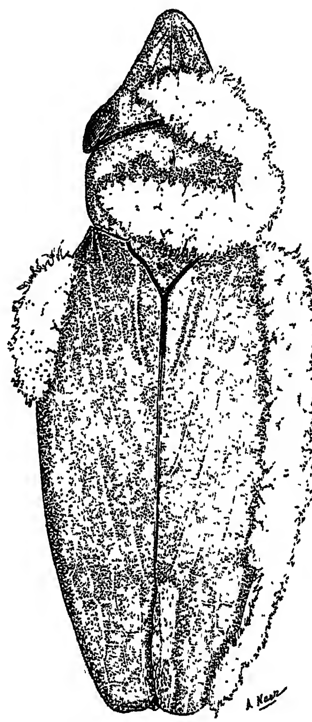


Fig. 100. A leafhopper, *Draeculacephala minerva* Ball, infected with *Entomophthora sphaerosperma* Fres.

culture media as used by Sawyer. Resting spores are formed asexually (azygospores), their formation being subject to such factors as temperature and the nature of the artificial media upon which the fungus is being grown.

Although *Entomophthora sphaerosperma* is distributed widely and on many different host species, detailed studies of epizootics caused by this fungus have been made in relatively few instances. One such study was accomplished in South Africa by Ullyett and Schonken (1940) in the case of such an epizootic among larvae of the diamondback moth, *Plutella maculipennis* Curtis, which are first miners and later surface feeders on cruciferous plants. These workers observed the sporadic occurrence of the disease in fields in Transvaal where, at times, it was an important factor in the temporary control of the insect.

During the early stages of the infection the *Plutella* larva shows no significant external symptoms. As the disease becomes more advanced, however, the insect becomes restless and changes in color from green to a yellowish green. The yellow color becomes intensified, and the larva becomes increasingly sluggish in its activity as the disease progresses. With the approach of death, the body becomes somewhat distended; and immediately after death and before the aerial hyphae appear the larva is extended on the leaf and is turgid and brittle. Upon being lifted, the body breaks easily, but there is little or no liberation of fluids. This last feature is a convenient one for making field diagnoses. Early larval instars appear to be more resistant to infection than do the later ones, fully grown larvae being very susceptible. The development of the fungus in the body of the larva is essentially the same as has been described as characteristic of the group. Germination of the conidia requires prior contact of the latter with free moisture. Thus it is that epizootics of the disease break out only after heavy rains. The germination hypha penetrates the integument of the host and enters its body cavity where growth rapidly follows, giving rise to a branching mycelium. Infection of the *Plutella* larva almost invariably takes place at the anal extremity, the hyphal threads running forward longitudinally in the body cavity. This forward growth of the mycelium apparently is a normal attribute of the fungus, since regardless of where experimental infection takes place the initial growth is toward the cephalic end of the insect, taking place against the flow of the blood stream. The body cavity gradually becomes filled with the mycelium. Just before the death of the host the hyphae break up into short lengths, and immediately after death the mycelium is a compact mass of short stout lengths of hyphae (the hyphal bodies) which completely fill the body of the insect. The reproductive phase of the fungus begins with the death of the insect. Rhizoids anchor the dead larva to the leaf or other substratum, and hyphal branches emerge and ramify over the surface becom-

ing interwoven at right angles to one another until the body is covered with a grayish-white felt of fructifying mycelium. Branched conidiophores arise from the thallus thus formed and bear the characteristic elongate conidia. When the mature conidia are discharged they come to lie on the leaf surface immediately surrounding the dead insect. It is from this source that healthy larvae pick up the spores as they crawl over

Fig. 101. European apple sucker, *Psyllia mali* Schmidb., killed by *Entomophthora sphaerosperma* Fres. Conidial stage of the disease. (*From Dustan, 1924; courtesy of Dominion Department of Agriculture, Division of Entomology.*)

them. At the completion of the aerial fruiting of the fungus, the center of the thallus turns brown in color. Within the body of the dead host azygospores are formed from the hyphal bodies. These spores remain within the mummified host until it becomes broken up or until favorable weather conditions induce germination. Under dry conditions the viability of the azygospores is retained for long periods of time.

The effect of epizootics caused by *Entomophthora sphaerosperma* on the *Plutella maculipennis* population was examined by Ullyett and Schonken (1940) from both the theoretical and the practical standpoint. The details of their observations and conclusions in this regard will be considered in Chap. 14. In brief, however, these workers concluded that the fungus, although it produced a decided immediate reduction in the host population during the favorable weather conditions under which it acted, was ulti-

mately responsible for an increase in the average density of the host and, through this, for the occurrence of economic damage to the crop. The principal reason for this situation appeared to be the concurrent destructive effect on the insect parasites and predators that normally held the population density low enough to avoid serious crop damage. Thus the intervention of the disease in an existing equilibrium system resulted in the destruction of permanent mortality factors (insect parasites and predators) and their replacement by a temporary mortality factor (the fungus). When the activity of the fungus ended, the host population was able to recover more rapidly than its normal parasites and thus to attain a higher density level than before. This is an excellent example of the fact that occasionally a destructive disease may actually be a detriment rather than an aid in the long-range control of an insect pest.

An instance in which the artificial distribution of *Entomophthora sphaerosperma* affected the localized control of a destructive insect is provided by the work of Dustan (1924), who used it against the European apple sucker, *Psyllia mali* Schmidb., in Nova Scotia. This host was first found infected in considerable numbers in 1920 and again in 1921, and in the humid years of 1922 and 1923 artificial distribution of the fungus practically exterminated the insect in certain orchards. The fungus was spread by pinning leaves bearing diseased insects to leaves in the orchard being treated and by the liberation into the orchards of infected adults still capable of flying about. About a week after such "plantings," the fungus began to appear on the apple suckers in the orchards being treated. It spread very rapidly and was generally distributed throughout the orchards within 2 or 3 weeks if weather conditions were favorable. Dustan was able to initiate outbreaks of the diseases earlier than would naturally be the case by building up active epizootics in caged material early in the season and then introducing this material into the orchards.

***Entomophthora aulicae* (Reich.).** The so-called "brown-tail fungus," *Entomophthora aulicae*, attacks the caterpillars of the brown-tail moth, *Nygmia phaeorrhoea* (Donov.), as well as more than a dozen other insect hosts. Thaxter (1888) considered this fungus as a strain of *Empusa grylli* (Fres.), but it has since been recognized as a distinct species. It has long been known in Europe and was first recorded in the United States by Thaxter in 1888. Both natural and artifical epizootics of the fungus on the brown-tail caterpillar have been reported.

In the spring and early summer the caterpillars leave their nests to feed on young leaves, and in the autumn they again leave before the webs of the new brood are closed for the winter. During both these periods, Speare and Colley (1912) found that the fungus could be effectively used against the insect, depending on the presence of favorable weather con-

ditions, which appear to consist of warm nights and damp atmosphere. The natural epizootics also occur during these periods and under essentially the same conditions.

After developing methods for the artificial propagation of *Entomophthora aulicae* on brown-tail caterpillars in cages, Speare and Colley came to the following conclusions with regard to their attempts to use the fungus against this insect in the field. They were able to effect the destruction of caterpillars in great numbers and over considerable areas, although the general effectiveness of this method was variable since the factors of temperature and humidity could not be controlled. Nevertheless the introduced disease could usually be depended on to kill from 60 to 100 per cent of the caterpillars in the areas where diseased individuals were distributed. Best results were obtained in localities where the disease was not known to occur naturally. The fungus usually lives over winter from the autumn infection and reappears early in the spring, reducing the caterpillar population. For this reason the autumn infection is doubly effective. Artificial distribution appears to be most easily accomplished in sprout woodlands and in pastures where ordinary methods of control (*e.g.*, spraying and cutting) are not employed and where the caterpillar nests are more readily accessible.

Speare and Colley's (1912) report also contains a description of experiments with an unidentified *Entomophthora* on larvae of the gypsy moth, *Porthetria dispar* (Linn.). They concluded that this entomophthorous disease was not a promising one for artificial use.

***Entomophthora fumosa* Speare.** It has long been recognized that in Florida the citrus mealybug, *Pseudococcus citri* Risso, is a pest of only secondary importance. In certain parts of California, on the other hand, it is considered one of the most serious enemies of citrus plants. The reason for this interesting difference was pointed out by Speare, who in 1922 published a revealing account on the natural control of the citrus mealybugs in Florida. According to Speare, the principal reason that *Pseudococcus citri* Risso is not an economically important pest in Florida is the fact that it is held in check by an entomogenous fungus, *Entomophthora fumosa*. The climatic conditions in Florida during the growing season are optimum for the development of the fungus, whereas this is not the case in California. In Florida the warm temperatures and the rainy season coincide; in California they do not—the rainy season occurs during the cooler winter months. Thus it is that in Florida the fungus has an opportunity to flourish and to bring about the natural destruction of its mealybug host.

Although Speare originally gave the fungus the generic name *Entomophthora*, it would probably be more appropriate for it to bear the name *Empusa*, since the conidiophores are of the simple type. This designation

depends, of course, upon the importance one is inclined to give the type of conidiophore in the generic separation of the Entomophthoraceae. The conidia of the fungus are more or less fusiform in shape, approximately 9 by 18 microns in size, and distinctly smoke-colored. Resting spores are formed and are usually spherical, black, and provided with a hyaline protuberance or appendage.

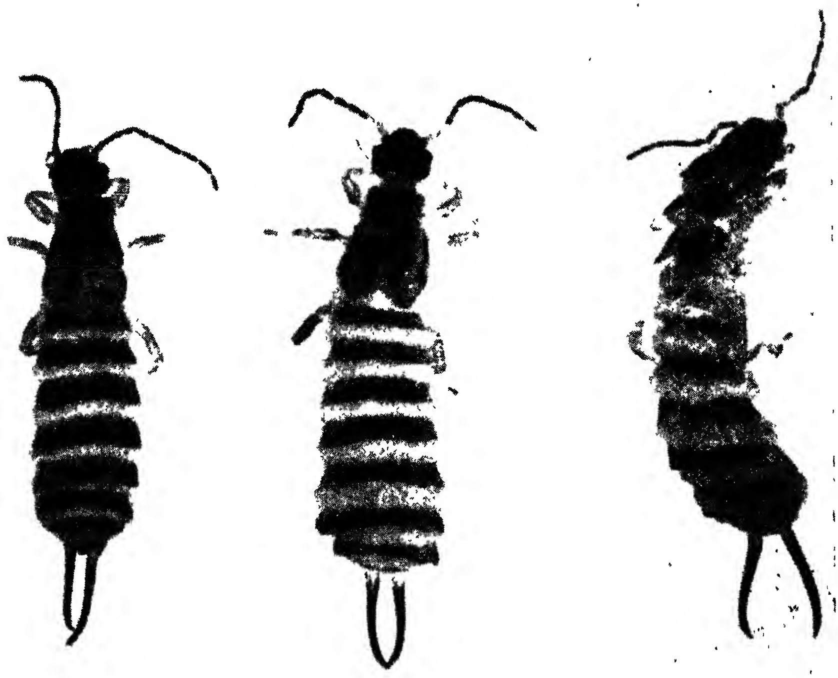


Fig. 102. The European earwig, *Forficula auricularia* Linn., killed by infection with *Entomophthora forficuli* Giard. The conidiophores of the fungus are emerging through the intersegmental areas. (*Photograph by Getzendaner; courtesy of B. J. Landis, U.S. Department of Agriculture.*)

The symptoms of the disease caused by *E. fumosa* have been described by Speare (1922). One of the earliest signs of infection may be elicited by the use of a penknife or other bluntly pointed instrument which, when pressed against the body of a healthy mealybug, creates a depression that quickly returns to its original position when the pressure is withdrawn, much as a rubber ball would do. In mealybugs in the early stages of the disease, however, the body wall ruptures very easily when pressed with a penknife, allowing a droplet of milky fluid to exude. This occurs in the infected insects, even though to all outward appearance they may seem

entirely healthy and active (at times their movements may be somewhat sluggish). If similar treatment is applied to a recently dead mealybug, which is also lifelike in appearance, the depressed area remains sunken. After the insect has been dead from 12 to 18 hours, its body is more or less solid to the touch, and if considerable pressure is applied to the penknife, the body will cut like a piece of cheese. No significant change in the external appearance of the mealybug takes place until about 24 hours after the first symptoms have been observed. At this time, and later, the infected insects may present one of two types of appearance. Most of the dead insects will appear to be enveloped in a dark slate-gray woolly covering, which consists of the fungal conidiophores and the developing conidia. A few of the insects, on the other hand, may appear jet black in color, sometimes almost glistening, with the body surface of the insect smooth instead of woolly. The jet-black color is due to the large numbers of spherical black resting spores formed within the body of the insect. The color of the black spores is transmitted through the thin translucent body wall still intact. In both of the two types of infection the mealybugs are attached rather lightly to the host plant by the insertion of their proboscises.

In orchards of grapefruit, Speare observed an extremely rapid spread of the fungous infection among the mealybugs during the months of June, July, and early August. In one grove, the percentage of mortality jumped from 18 to 64, an increase of 46 per cent, in but a week's time (June 22 to June 29). The role of the fungus in the natural control of mealybugs is also emphasized, in Speare's opinion, by the fact that following the application of fungicides, which destroy the entomogenous fungus as well as that of the citrus scab, the numbers of mealybugs rose sharply in the groves so treated.

Incidentally, certain Fungi Imperfecti have from time to time been reported as being parasitic on mealybugs. Species of *Aspergillus* and *Cephalosporium* have been mentioned in this connection. Boyce and Fawcett (1947), for example, relate that during the propagation of mealybugs in California insectaries, a fungus closely related to *Aspergillus parasiticus* Speare proved to be a potentially serious parasite under conditions of high humidity and moderate to high temperatures. Efficient ventilation of the insectaries, careful irrigation of the host plants, and a lowering of the temperature to 20°C., or below, are suggested control measures. A fungus that has been named *Endosclerotium pseudococcia* Har. & McK., occasionally kills large numbers of mealybugs, including the Comstock mealybug, *Pseudococcus comstocki* Kuw., in the apple orchards of Virginia and other eastern states. When conditions for growth are unfavorable, the fungus produces a highly resistant compound sclerotium which may remain viable several months.

Infection Caused by *Massospora*

Of the remaining genus of Entomophthoraceae, *Massospora*, the only well-studied entomogenous species is *Massospora cicadina* Pk., named and described by Peck in 1879, although apparently seen by Leidy as early as 1850. It is a parasite of the seventeen-years locust, or periodical cicada, *Magicicada septendecim* (Linn.), and had been observed particularly in the Eastern and Middle Western parts of the United States.

The occurrence of the fungus was subsequently recorded in the reports of a number of entomologists, but it remained for Speare (1921) to give the first adequate description of the disease itself, as well as that of the microscopic characters of the fungus. He also showed the relationship of *Massospora cicadina* to other entomogenous Entomophthorales. Goldstein's (1929) cytological study of the fungus furnished additional information on this very interesting parasite.

In some outbreaks at least, the fungus appears to be confined largely, although not exclusively, to male insects, and in the resting spore condition it usually parasitizes spent individuals, females as well as males having been found in this condition. It confines its vegetative growth to the softer tissues in the posterior segments of the cicada's body. Most of these tissues are completely destroyed, and as a result of the destruction of the flexible intersegmental membranes, the posterior abdominal segments, beginning with the last segment, slough off. The insect, however, remains alive for a considerable period, continuing to fly and crawl about. This unusual phenomenon is so striking that it is readily noticed by all observers.

One of the outstanding characteristics of *Massospora cicadina* is that, unlike species of *Empusa* and *Entomophthora*, it produces conidia within the body of its host rather than on its surface, where they are violently discharged from the conidiophores. Within the abdomen of the cicada, the conidia cohere with one another forming clumps, clusters, or a mass that is exposed when the insect's abdominal segments drop off. The movements of the insect then aid in scattering the conidia of the fungus. The conidia are oval in form, about 12 by 15 microns in size, and have a distinct but not prominent papilla. Unlike those of most other Entomophthorales, the conidial walls are regularly verrucose. Resting spores, found in certain individuals, are slightly brownish spherical bodies with reticulations that give them an appearance similar to the design on golf balls. Attempts to cultivate the fungus on artificial media have not been successful.

Just how or where the fungus survives the 16¾ years of the host's immature and subterranean existence is not known. It is possible that

infection takes place while the insect is underground, or the fungus may exist on species of biennial cicadas when its regular host is absent. Its importance in the natural control of the periodical cicada is probably not great, if Speare's observation that the infection is confined largely to spent males is correct. Goldstein (1929), however, found the fungus in both males and females, and most of her specimens containing resting spores were females whose bodies still contained many eggs. Such a situation would enhance the importance of the fungus from an economic viewpoint.

Infections Caused by Chytridiaceous and Blastocladiaceous Fungi

Among the more primitive of the phycomycetous fungi are those belonging to the orders Chytridiales and Blastocladiales. The "chytrids," as the former are commonly called, occur mainly in fresh water on a variety of substrata. A number of species live in the submerged and empty exuviae, or castoff integuments, of the immature stages of certain insects (Sparrow, 1937).

Truly entomogenous chytridiaceous fungi, however, have been reported by a number of mycologists. Wize (1904) found such a fungus in Coleoptera (*Cleonus*, *Anisoplia*) larvae and pupae collected in the Ukraine, and Sparrow (1939) reported on similar observations made on dipterous pupae collected in the United States by Thaxter. In both instances the fungus appears to have been the same, *Myiophagus ucrainicus* (Wize) [*Myrophagus*]. The body contents of the diseased insects are almost completely disintegrated and replaced by an orange to reddish mass of fungous material. What is apparently this same fungus has also been found in dipterous pupae in England (Petch, 1940), and in Bermuda, Canada, and the United States in scale insects (Waterson, 1946; Fisher, 1947; Karling, 1948). Its occurrence in scale insects (purple scale, chaff scale, oyster-shell scale, red scale, long scale, and soft brown scale) is particularly interesting and the chytridiosis produced probably deserves study from the standpoint of biological control. Purple scales have been infected by spraying them with aqueous suspensions of the fungus, and mealybugs have been found experimentally susceptible.

The entomogenous Blastocladiales are better known than are the apparently few chytrids that parasitize insects. Nevertheless these are confined largely to one group, the family Coelomomycetaceae, which parasitizes mainly mosquito larvae.

Coelomomyces Infections

The first recognized infection caused by a member of this group of fungi was that discovered by Keilin in 1921, occurring in larvae of the

mosquito *Aëdes albopictus* (Skuse) (= *Stegomyia scutellaris* Walker) collected in the Federated Malay States. To this fungus, which he believed to be related to the Chytridineae, Keilin gave the name *Coelomomyces stegomyiae*. The next year Bogoyavlensky (1922) described, under the name of *Zografia notonectae*, a fungus parasitic in the body cavity of *Notonecta* (Hemiptera) collected from ponds in Moscow. Keilin (1927), however, showed this organism to be of a nature similar to that of the fungus he studied, thus giving it the name *Coelomomyces notonectae*. Additional species have been isolated from mosquitoes (*Anopheles*, *Culex*, *Psorophora*, and *Uranotaenia*) in other parts of the world, including 2 species from India (Iyengar, 1935), 1 species from Africa (Walker, 1938; Haddow, 1942), and 11 species and 2 varieties from the United States (Couch, 1945; Couch and Dodge, 1947).

Fig. 103. Head and thorax of a larva of *Anopheles quadrimaculatus* Say, containing oval resting sporangia of *Coelomomyces dodgei* Couch. (*From Couch, 1945.*)

The Infection. The manner in which mosquito larvae become infected with *Coelomomyces* has not been determined with certainty; *i.e.*, it is not clear whether invasion takes place via the digestive tract following the ingestion of the zoospores or the sporangia, or directly through the insect's integument. In any case the principal development of the fungus occurs within the body cavity of its host. Depending upon how far the infection has progressed, only certain regions of the body, such as the gills or the posterior segments, may appear involved, or the entire hemocoele may appear filled with the parasite. Frequently the mycelia and spores of the fungus may occur even within the head of the larva.

The hemocoele of an infected larva in the fourth instar may appear as a solid mass of sporangia, which gives the larva an opaque aspect. The color of such larvae may be dull or yellowish-white, bright yellow, orange, or even a dull reddish-brown.

In most instances the fungus completes its development during the larval stage of the mosquito. Sometimes, however, when infection occurs late in larval life the fungus completes its development in or persists through the pupal and adult stages. In surviving adult females the infection is usually confined to the ovaries. In other cases it occurs throughout the body of the adult, causing its death. Infected pupae may be

unable to emerge as adults; especially is this so once the sporangia have developed. It is probable that the majority of infections begin during the larva's first instar, since fully developed sporangia are found in the third and fourth larval instars. Since this period of larval development is usually one of only 6 to 10 days, it indicates the very rapid development of the fungus. Actually, under normal conditions in nature, it takes only

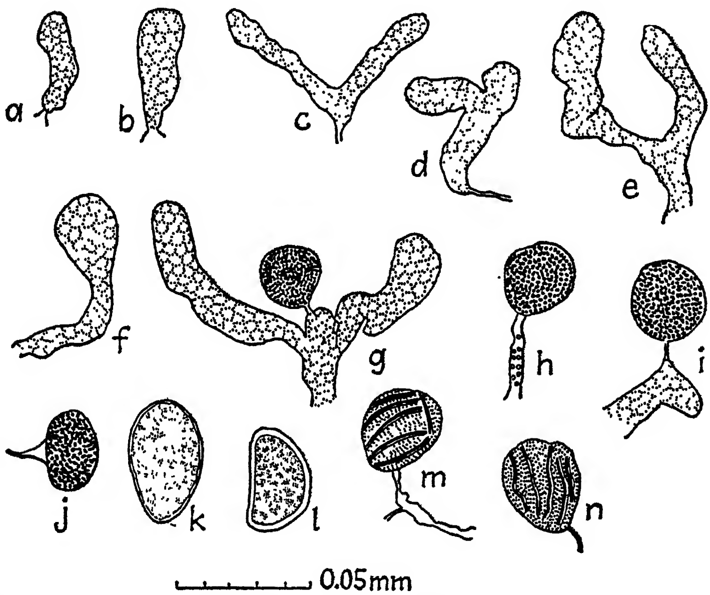


Fig. 104. *Coelomomyces anophelesica* Iyengar. *a* and *b*, young mycelia; *c–f*, older mycelia; *g* and *i*, formation of sporangium; *h*, formation of sporangial wall and withdrawing of contents of mycelium; *j*, young sporangium with attached strand; *k*, vacuolated stage of sporangium; *l*, older sporangium with dense contents and thick wall; *m* and *n*, mature sporangia with partly developed ribs and connecting strand. (*From Iyengar, 1935.*)

2 or 3 days for the thalli to develop into sporangia. According to Iyengar (1935), infection generally begins in the thoracic region of the larva and spreads posteriorly into the abdominal segments, traveling along the adipose tissue on which the fungus lives. As a result of the attack by the fungus, the fat body loses its characteristic appearance and shrinks, the nuclei of the fat cells disintegrate, and finally the fat body completely disappears. In place of the fat body, a thin membrane filled with many dark-brown pigment granules remains. Only rarely does an infected larva of the fourth instar have any fat tissue. Some fat tissue may remain if the infection has been of a low intensity. One reason for an infected larva's rarely completing its metamorphosis into an adult mosquito is the suppression of the imaginal buds by the infection. In a fourth-instar infected

larva, the wing and leg rudiments are underdeveloped as compared with their appearance in a healthy larva of the same instar.

No accurate survey has yet been made as to the over-all extent to which mosquito larvae are infected with *Coelomomyces* fungi. In some areas the incidence of infection appears to be very high, while in other areas infected specimens can be found only on rare occasions. Muspratt (1946*a*) estimates the mortality, in pools that were under his observation in Rhodesia from 1941 to 1945, to be as high as 95 per cent of the larvae that hatched from eggs during the rainy season. Of those larvae which reached the fourth instar, at least 9 out of 10 were infected and subsequently died. Offhand, at any rate, such data would make it appear that these fungi may possibly have some use in the biological control of mosquitoes, at least in the tropics. On the other hand, some observers (*e.g.*, Couch and Dodge, 1947) have found the ratio of infected larvae to healthy ones to be so small in the areas they studied that the fungus did not appear to have much importance as a natural control agent. The biological control possibilities are under consideration by several groups of investigators as this is being written, and the outcome of these as well as other studies must be awaited before it will be safe to draw any definite conclusions in this regard.

The Fungi. As has already been mentioned, the *Coelomomyces* fungi are Phycomycetes belonging to the family Coelomomycetaceae of the order Blastocladiales. It was at first believed (Couch, 1945; Muspratt, 1946*b*) that, like those of most species of Blastocladiales, the resting bodies of *Coelomomyces* required a period of desiccation before being able to germinate. Muspratt goes further to suggest that in order to bring about the germination of the resting sporangia, it may be necessary to use rain water and allow it to evaporate in the sun to about one-third of its volume before infection can be expected, the germination perhaps being regulated by a slight increase in the concentration of the soil mineral salts in solution. In 1947, however, Couch and Dodge found germination to occur in the case of three species without the previous drying of the resting bodies. These authors nevertheless feel that the fungi belong to the order Blastocladiales, particularly since in structure and method of swimming the zoospores are typical of this order.

Germination of the sporangia was first witnessed by De Meillon and Muspratt (1943). These workers observed the process to be somewhat as follows: When about to germinate, a sporangium first loses its oil droplets and the interior becomes granular. A slight bulge then appears on one side. As the bulge enlarges, the outer hard shell ruptures and two thin internal membranes appear. The content of the sporangium flows out and is confined within the innermost of the thin membranes, both

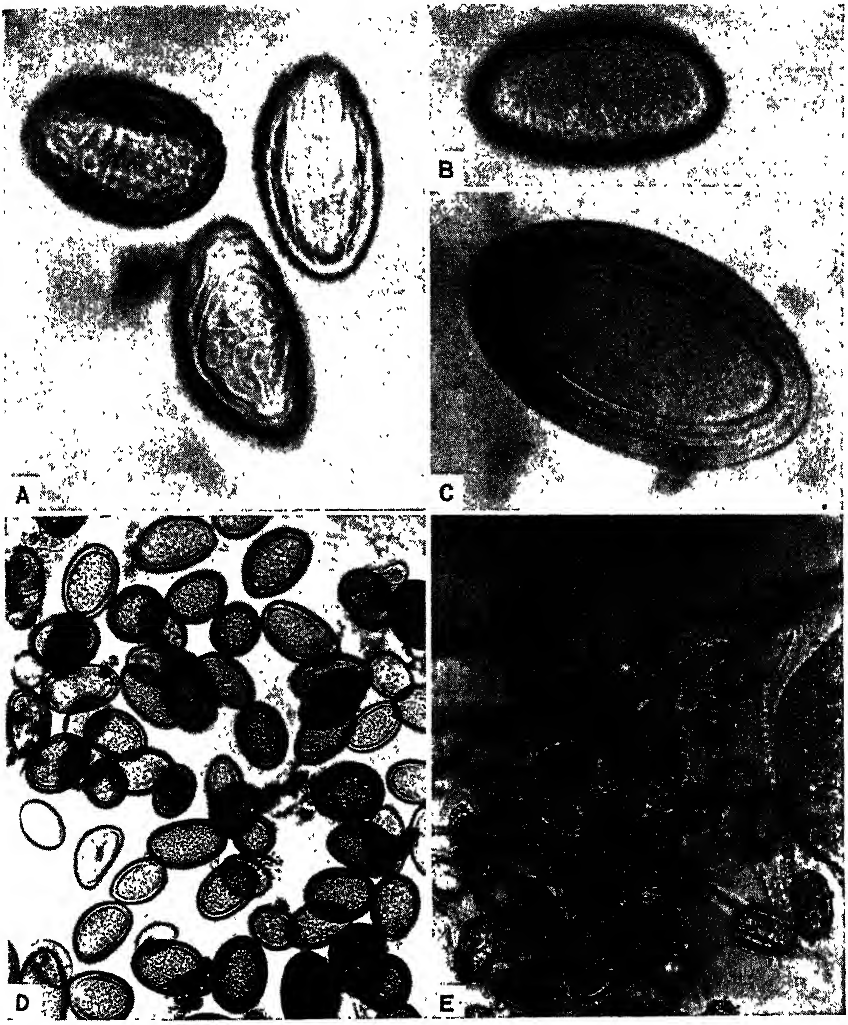


Fig. 105. Resting sporangia of *Coelomomyces*. Magnified. *A. C. lativittatus* Couch & Dodge from the mosquito *Anopheles crucians* Wied., showing wide bands. *B. C. punctatus* Couch & Dodge from *Anopheles quadrimaculatus* Say. *C.* Longitudinal section of mature resting sporangium of *C. keilini* Couch & Dodge from *Anopheles crucians* Wied. *D. C. psorophorae* Couch from *Psorophora ciliata* (Fabr.). *E. C. uranotaeniae* Couch from *Uranotaenia sapphirina* (Ost.). (*From Couch, 1945; Couch and Dodge, 1947. Courtesy of J. N. Couch.*)

of which become increasingly more visible and more widely separated. The contained zoospores become less densely packed and accordingly begin to move about with increasing speed until the interior of the sporangium, and the extruded membranes, are a seething mass of zoospores. Within a few minutes thereafter the zoospores find their way out to the exterior until the sporangium and rent membranes are empty. This whole process may take a day or two; but once the zoospores flow into the

Fig. 106. Mycelium and a few resting sporangia of *Coelomomyces quadrangulatus* var. *irregularis* Couch & Dodge from *Anopheles punctipennis* (Say). (*From Couch and Dodge, 1947.*)

extruded membranes, complete liberation takes place in a few minutes. The zoospores have single flagella about four times the length of the zoospores, which measure approximately 4 microns.

Further observations on the germination of the resting sporangia were reported in 1947 by Couch and Dodge. They also observed germination to begin with a swelling of the contents which causes the outer wall to split along a preformed line. The contents continue to swell, bulging out through the fissure to form a dome-shaped mass surrounded by the inner spore wall. The zoospores are formed 24 to 36 hours later, and the entire exposed part of the inner wall quickly swells and dissolves to set the zoospores free. The two thin membranes described by De Meillon and Muspratt are interpreted by Couch and Dodge as a single membrane that gelatinizes. Otherwise the observations of these two pairs of workers are essentially similar.

Within the body cavity of the insect, the fungus develops a coenocytic, aseptate mycelium without rhizoids or cell walls, and surrounded only by a plasma membrane. This last characterisic enables the fungus to absorb its food directly over its entire surface. The hyphae are irregularly or rarely dichotomously branched and consist of single threads of more or less uniform diameter except at certain points. The hyphae give rise to irregularly shaped hyphal segments that are severed from the parent hyphae by division or by the thinning down of the basal connection until it is broken away by the movements of the insect's body. The sporangia are formed as are the hyphal segments. They break away from the mycelium before the formation of a definite wall and complete their development within the hemocoele of the insect. The thick-walled sporangia fill the body cavity and are usually set free upon the disintegration of the insect's body. The sporangium is surrounded by an exceedingly thin, hyaline, smooth, outer membrane derived from the old plasma membrane. The wall is two-layered, the outer layer usually being the thicker. It may be smooth, pitted, banded, striated, ridged, or otherwise sculptured or ornamented. Dehiscing occurs by a preformed longitudinal slit. All these characteristics have been stated descriptively by Couch (1945), who revised the genus *Coelomomyces* Keilin and erected the family Coelomomycetaceae.

ASCOMYCETE AND DEUTEROMYCETE (FUNGI IMPERFECTI) INFECTIONS

It is more for convenience than for any other reason that we place the ascomycete and deuteromycete infections under one general heading. Most authorities recognize the majority of species now relegated to the class Deuteromycetes (Fungi Imperfecti) to be, in reality, species of Ascomycetes whose relation with an ascogenous stage has not been established. In other words, since most of the Deuteromycetes appear to be asexual (imperfect) stages of recognized or unrecognized species of Ascomycetes, it is expedient for us to consider the two groups together. Especially is this a convenient procedure in dealing with such entomogenous fungi as those which parasitize scale insects and whiteflies and which, in many cases, are known by different names in both their sexual and asexual (perfect and imperfect) stages.

As has been expressed by Wolf and Wolf (1947), the Ascomycetes "possess a multiplicity and complexity of architectural design and a seemingly infinite variety of patterns of activity that are baffling to all who attempt to catalogue or to orient them." It is impossible, therefore, to present this large group according to any one system of classification and nomenclature, and at the same time to satisfy all systematic mycolo-

gists. For the present, the best that the insect pathologist can do is to follow the lead of those who have given the greatest attention to these entomogenous fungi until some authority makes it clear that the wrong name is being used or that the fungus is in an incorrect taxonomic location. We shall attempt to follow such a procedure in the present discussion and, inasmuch as is possible, spare the reader details of the annoying controversies or the equally trying indifference that prevail among specialists in some groups of the fungi concerned. This applies to both the Ascomycetes and the Deuteromyctes (Fungi Imperfecti). In the latter group, of course, the groupings are admittedly artificial and without real phylogenetic significance.

The Ascomycetes are characterized by the formation, somewhere in the life history, of a definite number of spores (ascospores), usually eight, contained within a unicellular sac or membrane (ascus). The ascospores are formed as the result of sexual fusion of nuclei. In addition, many Ascomycetes also produce conidia, or exogenous asexual spores, borne on stalks of mycelium known as "conidiophores." As a rule, conidia are produced in large numbers when conditions are favorable for the rapid multiplication of the fungus. Many species form ascospores in only small numbers and frequently under particular and uncommon circumstances. The hyphae of Ascomycetes are generally septate, and cells thus formed are usually uninucleate.

Most authorities consider the Ascomycetes as consisting of two subclasses, Hemiascomycetes and Euascomycetes. In the first of these, which is the more primitive, the asci occur singly or in groups, but ascocarps are lacking. In the second, which includes most of the Ascomycetes, the asci are aggregated and ascocarps are present. Ascocarps are special large fruiting organs in which the asci are formed. The ascocarp may be arranged as either a widely opened, cup- or saucer-shaped structure (the apothecium), or as a hollow sphere (the perithecium) usually having a small apical opening, or ostiolum, through which the ascospores escape at maturity. Variations of these two types occur (*e.g.*, cleistothecium and hysterothecium) but such are rare among entomogenous fungi.

Infections Caused by Yeasts

Of the subclass Hemiascomycetes, the order Endomycetales (Saccharomycetales), commonly called "yeasts," is known to contain a few species pathogenic for insects. As early as 1879 the Harvard entomologist Hagen advocated the use of beer mash, or what he termed a "yeast fungus," for the control of grasshoppers, potato beetles, phylloxerids, and other noxious insects. However, no actual attempt to control an insect in the field through the use of a yeast has been recorded. Several

saprophytic yeasts have been isolated from insects, and a few have been described as infecting small groups of insects.

The first adequately described yeast infection of insects appears to have been that discovered in England by Keilin (1920) in larvae of the biting midge, *Dasyhelea obscura* Winnertz, a dipteran insect living usually

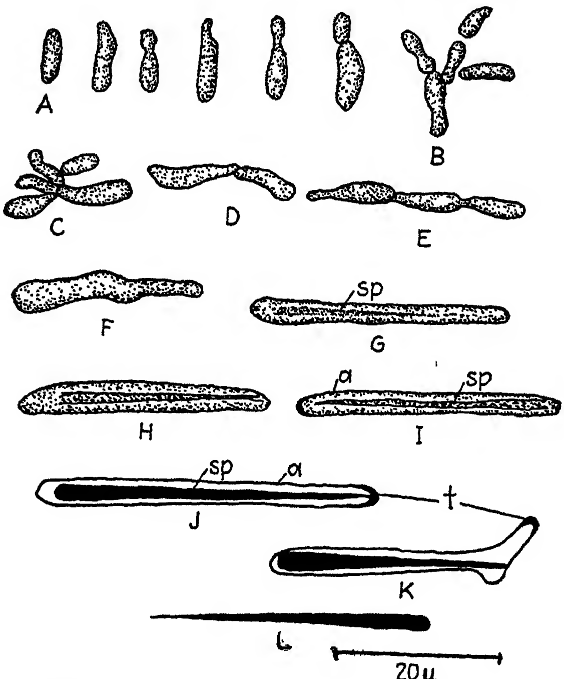


Fig. 107. *Monosporella unicuspidata* Keil. from the larva of *Dasyhelea obscura* Winnertz. *A–B*. Different stages of budding cells. *B* and *C*. Rare cases of multiple budding. *D*. Ordinary budding. *E*. Chain with three cells. *F*. Elongated cell developing into ascus. *G*. Ascus with beginning of spore formation. *H* and *I*. More advanced stages of spore formation. *J*. Ascus (*a*) with well-formed spore (*sp*); *t*, thickened wall of the ascus. *K*. Deformed ascus. *L*. Spore. (*Redrawn from Keilin, 1920.*)

in the sap that fills infected wounds of elm or horse-chestnut trees. The infecting yeast, *Monosporella unicuspidata* Keil., invades the body cavity, which becomes so filled with the parasite as to give the insect's body, especially its posterior segments, a milky appearance. Even though a great number of parasites is present, the larva is able to move until it finally dies and decomposes rapidly, setting free the resistant forms of the yeast. The fat body is apparently the only organ that is completely destroyed.

The yeast itself occurs in several forms. Young organisms appear as

small oval cells, from 4 to 10 microns long, budding at one end. Later, the parasites become elongated until they reach a size of about 30 microns in length by 2.5 microns in width. These elongated forms constitute the asci, in each of which develops a long needle-shaped unicellular spore with one end sharply pointed. It is assumed that this truncated end assists the spore in penetrating through the gut wall of the insect during early stages of invasion, in a manner analogous to that observed earlier by Metchnikoff in the case of a similar organism infecting a crustacean (*Daphnia*).

A yeastlike organism, which they named *Mycoderma clayi*, was isolated in 1928 by Metalnikov, Ellinger, and Chorine from European corn-borer larvae shipped to them from Canada. The organism appeared as a large gram-positive rod, measuring 9 to 16 microns in length by 1.5 to 3 microns in width. Propagation occurs by budding. The yeast is readily cultivable and to a slight degree ferments glucose, levulose, sucrose, and glycerin. When injected into the body cavity of the larva, the cells multiply rapidly, causing a septicemia that kills the insect in from 2 to 5 days. Both in vivo and in vitro the organism has a tendency to form mycelium. Spores have not been observed.

Another *Mycoderma*, *M. cerevisiae* (Desm.) was cited by Burnside (1930) along with *Saccharomyces cerevisiae* Hansen and *S. ellipsoideus* Hansen as causing a dysenteric condition among caged bees that were heavily inoculated by feeding. Sometimes a condition resembling intoxication developed, from which the bees rapidly recovered. When these yeasts were inoculated directly into the body cavities of bees, death resulted in from 50 to 100 per cent of the cases. With *Saccharomyces apiculatus* Hansen death occurred in about 50 per cent of the individuals similarly inoculated. Microscopic examinations of the diseased bees showed that the yeast cells multiplied rapidly in the blood and at the time of death were present in such large numbers that the blood took on a milky appearance. The organisms appear to develop most abundantly on the muscle fibers of the thorax. The presence of a chalk-white coating on the surface of the muscles after they have dried was one of the most distinctive macroscopic symptoms of these yeast infections. Lardinois (1926) observed *S. apiculatus* constantly associated with lesions in the tissues of bees afflicted with constipation and expressed the belief that this yeast is the sole cause of "May disease."

In an attempt to find a microorganism that might be used in the control of mealybugs in Russia, Evlakhova (1939) reared *Pseudococcus citri* Risso in the laboratory under conditions of high humidity to favor the development of disease organisms. Among those isolated was a yeastlike fungus which he named *Blastodendrion pseudococci*. Under experimental

conditions of 25°C. and 60 to 70 per cent humidity, a mortality of mealybugs occurred ranging up to 50 per cent; with cultures of increased virulence a mortality of as high as 100 per cent was reached. Most of the mealybugs died in 24 hours after commencing to feed on potato stems smeared with the cultures. Spraying the mealybugs with a suspension of the organism was less effective. In causing the infection, the yeast apparently entered the insect's body through the digestive tract, penetrating into the fat body and the muscular system.

An interesting pathology involving a yeast has been described by Frobisher (1926) in *Drosophila melanogaster* Meig. being reared in the laboratory. The yeast concerned was a red torula, which was being used as food by the drosophila flies. Occasionally there appeared in the flasks used for cultivation a blue-green *Penicillium* which was capable of infecting and killing the flies. Besides the actual invasion of the insect tissues by the fungus, another mechanism seemed to be responsible for the death of the insects. The fungus appeared to grow among the torula cells in the gut and by matting them together formed a more or less solid mass that could block both elimination and engorgement. By invasion of the intestinal wall, the fungus hyphae were believed to bind this mass firmly in the intestine.

Hypocreales Infections

The order Hypocreales belongs to the subclass Euascomycetes and contains several genera noted for their entomogenous species. These genera fall into rather heterogeneous groups, which are nevertheless convenient for purposes of discussion. It is for this reason that we shall consider as a group the fungi parasitic on scale insects and those parasitic on whiteflies, even though some of the fungi coming under these categories are not Hypocreales. Some Hypocreales, such as *Cordyceps*, are clearly defined as taxonomic groups and may be discussed as such.

Cordyceps Infections

The genus *Cordyceps* contains about 200 known species, nearly all of which parasitize insects. Almost 40 recognized species occur in the United States. These have been studied principally from a taxonomic standpoint (*e.g.*, by Mains, 1939 *et circ.*). Some species originally considered as *Cordyceps* are placed by some authors (*e.g.*, Petch, 1931; but not now recognized by Mains, 1948) in the genus *Ophiocordyceps* which differs from the former in having clavate asci and fused overlapping ascospores. Cordyceps are cosmopolitan in distribution and occur on representatives of several orders of insects, principally Hemiptera, Diptera, Lepidoptera, Hymen-

Fig. 108. Insects parasitized by species of *Cordyceps*. *A*. *Cordyceps ravenelii* Berk. & Curt. on the larva of a June beetle. *B*. *Cordyceps amazonica* P. Henn. on a cockroach. *C*. *Cordyceps viperina* Mains on the larva of a beetle. *D*. *Cordyceps curculionum* (Tul.) on an adult curculio beetle. (*Courtesy of E. B. Mains; from Mains, 1937, 1940, 1941.*)

optera, and Coleoptera. The larval stage appears to be the most frequent host, but different species may occur on every stage of insect development. Information concerning the identity of the various hosts is frequently meager because of the destructive or obliterating effect the fungus has on

the insect and because of the tendency early mycologists had of reporting the fungus simply on a "caterpillar," "pupa," "moth," and the like.

Associated with the genus *Cordyceps* are numerous interesting historical records. Early writers (*e.g.*, Gray, 1858; Cooke, 1892) have presented rather extensive accounts of the group as it was known to them at that time. Insects parasitized by these fungi were frequently known as "vegetable wasps" and "plant worms" (or "awetos" in New Zealand). Among the most celebrated of the "vegetable wasps" were those (*Vespa* and *Polybia*) parasitized by *Cordyceps sphecocephala* (Klotzsch). An account exists in which Torrubia, a Franciscan friar, tells of finding, in 1749, some dead wasps in a field near Havana and "from the belly of every wasp a plant germinated, which grows about five spans high." As recounted by Gray, Torrubia gave a representation of two wasps lying on the ground with a tree growing out of the base of each abdomen, while three other wasps are flying (!) around these trees, each flying insect having a similar tree affixed to it. These "trees" in all probability represent species of *Cordyceps*. On this basis it may be considered that the earliest known record of an entomogenous fungus was that made by Christian Paulinus in the beginning of the eighteenth century when he wrote that "certain trees in the island Sombrero in the East Indies have large worms attached to them under ground, in the place of roots." For additional curios of this type of historical information the reader is referred to Gray's (1858) privately printed "Notices of Insects That Are Known to Form the Bases of Fungoid Parasites."

Also of interest is the fact that some *Cordyceps*-infected insects are used as human food in certain parts of the world. Hoffmann (1947) tells of this as follows:

> Hepialid and other caterpillars are commonly found infected with fungus of the genus *Cordyceps*. Szechwan Province, China, is famous for this material and from here the caterpillars with fungus are sent to various provinces in China and abroad as well. About a dozen of the infected caterpillars, each with a long strand of fungal growth, are tied into neat bundles of uniform size. The shriveled caterpillar with a fungal filament longer than its own body is somewhat reminiscent of a rat-tailed maggot. These caterpillars are considered a tonic food and are made into a broth—both the caterpillars and the broth being consumed. These caterpillars are expensive with the result that only the middle classes or the well-to-do can afford to eat them as a delicacy or as tonic food. I have sampled this material myself and found it quite tasty, but since I felt fine both before and after doing so, I cannot testify as to its efficacy.
>
> This or related species of *Cordyceps* attacks insects other than caterpillars. I once knew of three peasants in the Canton area who had a large number of fresh cicada nymphs infected with *Cordyceps*. These were being sold as medicine but

they were unable to sell all of their supply so decided to have a feast on the remainder. The next few days they spent in the hospital as very sick men. Dry cicada skins are used extensively in old style Chinese medicine, but this was my first knowledge of the entire nymph, plus the *Cordyceps*, being so used.

Morphological Characteristics. The feature that characterizes the genus *Cordyceps* is the fact that the stroma arises from a sclerotium formed within the body of the insect on which the fungus is parasitic. As described by Massee (1895), who monographed the genus, this stroma usually

Fig. 109. Two types of perithecia arrangement. *A*. Superficial perithecia of *Cordyceps michiganensis* Mains. *B*. Portion of the head of *Cordyceps unilateralis* (Tul.) showing embedded perithecia. (*From Mains, 1934, 1939a.*)

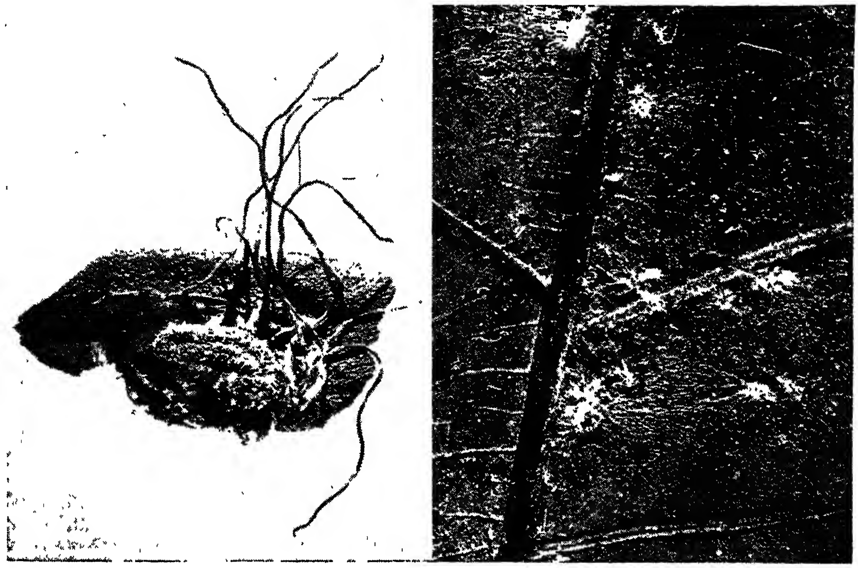
consists of an erect stemlike sterile portion composed of a fascicle of irregularly parallel hyphae, white internally, the external or cortical hyphae being usually tinged with color and in many species giving off numerous short lateral branches that form the minutely velvety or downy exterior of the stem.

The "head" or "club," which is the fertile portion of the stem, is usually terminal in location and may be brightly colored. The perithecia originate side by side deep in the stroma, their shape ovate or flask-shaped, their mouths reaching the surface of the stroma. They may remain completely immersed or at maturity be quite superficial, with the entire perithecium being exposed. The latter condition causes the surface of the head to be rough, whereas when the perithecia are immersed it is smooth.

The asci contain eight spores and are very long and slender. As the spores mature, the contents of the head become swollen, and the wall of the ascus is ruptured at the apex. The slender spores, arranged in a parallel fascicle, are almost as long as the ascus, are hyaline and multi-

septate or continuous. After escaping from the ascus, the multiseptate spores usually break up into their component cells, which eventually germinate. Paraphyses are absent.

Some species of *Cordyceps* are believed to have their conidial stages in such genera as *Isaria* and *Botrytis*. Such supposed relationships have not, however, been made too clear. For example, *Isaria farinosa* (Dicks)



A B

Fig. 110. Examples of *Hirsutella*. *A*. Codling-moth larva (*Carpocapsa pomonella* (Linn.)) infected with *Hirsutella subulata* Petch and showing the development of clavae. *B*. Part of a leaf (*Heveae*) showing lace bugs (*Leptopharsa heveae* D. & P.) attacked by the fungus *Hirsutella verticillioides* Charles. (*From Charles, 1937; courtesy of Bureau of Plant Industry.*)

(or *Spicaria farinosa* (Fron.)) was for years considered to be the conidial stage of *Cordyceps militaris* (Lk.). This belief and its supposed proof were discounted by Petch (1936), who contends that the conidial stage of this *Cordyceps* is a *Cephalosporium*. *Isaria farinosa* is a fasciculate *Spicaria* and may occur as an *Isaria* or as a simple *Spicaria*. It has no relation to *Cordyceps militaris*, which attacks larvae and pupae of Lepidoptera (and possibly Coleoptera; Petch, 1942), while *Isaria farinosa* is a general entomophyte occurring on Lepidoptera, Hymenoptera, Coleoptera, Diptera, Aphididae, and Arachnida. As Petch pointed out earlier (1934), the genus *Isaria* is an unsatisfactory one from several standpoints. If it is retained, it is a compound *Spicaria*. Petch suggests that it would be simpler to discard the genus *Isaria* and retain the name

as purely a descriptive term. A relationship between the imperfect genus *Hirsutella*, once considered a basidiomycete, and certain species of *Cordyceps* has been suggested by Speare (1920*b*), and supported by Petch (1923), who reduced his genus *Trichosterigma* to synonymy with *Hirsutella*. Since then several species of *Hirsutella* have been found to be the conidial stages of species of *Cordyceps*. Such may eventually be found to be the case with other species of *Hirsutella*, but many identities as to their ascigerous stages still remain uncertain (*e.g.*, *H. subulata* Petch on the larva of the codling moth, *Carpocapsa pomonella* Linn., and *H. saussurei* (Cke.), a parasite on hornets in the tropics, and others). Other genera of Hypocreales may, however, be related to *Hirsutella*. Species of *Calonectria*, for example, parasitize leafhoppers and are the ascigerous stage of certain species of *Hirsutella*.

The first to describe the growth of any species of *Cordyceps* from spore to spore in the laboratory appears to have been Shanor (1936), who accomplished this with *Cordyceps militaris*. Living lepidopterous pupae were inoculated with hyphae and placed in moist sterile sphagnum moss. Normal mature fruiting bodies were consistently produced. No success in producing perithecia has been obtained using artifical culture media.

Pathogenesis. To illustrate the life history of most *Cordyceps* and the manner in which they infect their hosts, we might use, as did De Bary (1887), the well-known *C. militaris* as an example.

After being ejected from an ascus of the orange-colored club-shaped stroma, the slender filiform, or rod-shaped spore, divides transversely into a large number of secondary spores. When one of these lands on the slightly moist skin of the host caterpillar, it swells slightly, becomes rounded in shape and puts out a germ tube. Sometimes the spores become partly united again by means of short connecting tubes before they germinate.

The germ tube proceeds at once to penetrate the integument, within which it enlarges into a somewhat thicker fungous hypha which ramifies its way into the deeper layers of the skin. It finally breaks through into the body cavity of the insect, insinuating itself between the muscular and fat tissue. Here the hypha breaks down into cylindrical bodies ("cylinder-gonidia") similar to the hyphal bodies already described in the case of the entomophthoraceous fungi. They continue to pass into the blood of the insect, where they elongate to twice or several times their original size, dividing repeatedly by transverse walls and by terminal and lateral sprouts. These cells disperse through the blood of the host until the body cavity gradually becomes filled with them as the quantity of blood is diminished. At this point the larva loses its normal turgidity, becomes soft and relaxed, and dies.

Fig. 111. *Cordyceps* parasitizing insects. *A. Cordyceps dipterigena* Berk. & Br. on a fly. *B. Cordyceps stylophora* Berk. & Br. on the larva of a beetle. *C. Cordyceps* [*Ophiocordyceps*] *clavulata* (Schiv.). *D* and *E. Cordyceps unilateralis* (Tul.) on ants. (*Courtesy of E. B. Mains; from Mains, 1939b, 1941.*)

As soon as the insect dies, the sprout cells begin to develop rapidly at the expense of the dead body tissues, forming branched hyphae, which fill the entire body cavity except the alimentary canal and expand the body to its former size and turgidity. The various tissues of the body are more or less absorbed by the fungus, and in 1 or 2 days a body is formed that retains the approximate size and shape of the living insect but consists essentially of a mass of fungous hyphae with some remains of the insect. This fungus body has all the properties of a sclerotium and may be considered as such. If this sclerotium lies in a moist situation, it can give rise directly to fresh stromata; if dried, it may lie dormant for several months.

The development of most *Cordyceps*, in their simplest form, follows much the same course as the one just described by *C. militaris*. Nevertheless variations occur between species and, in fact, within the same species. The life histories of species of *Ophiocordyceps* in most respects are similar to those of *Cordyceps* species.

Fungi Parasitic on Scale Insects

Some of the most notable work on the fungous diseases of insects has concerned those fungi parasitic on scale insects. Not all these fungi belong to the order Hypocreales, although many of the most important ones do. Some are known only in their imperfect or conidial stages. Since those which do belong to Hypocreales have been favored with a great amount of detailed study, it is considered convenient to use this order as a center about which all the fungi parasitic on scale insects may be considered.

According to Petch (1921), the earliest record of a fungus parasitic on a scale insect was made in 1848 by Desmazières, who collected his specimens from willow and ash at Caen, France. For many years following this, mycologists (including such systematists as Berkeley, the Tulasnes, Saccardo, and Petch) concerned themselves almost exclusively with the taxonomic aspects of these fungi. Eventually it was realized that at least some of these fungi may constitute an important natural check on certain species of scale insects. Beginning about 1912 considerable attention was focused on them in the citrus-growing areas of Florida, and attempts were made to use the fungi in the biological control of the insects (see Chap. 14). For a time it was believed that the increase of scale insects following Bordeaux spraying was the result of killing off the entomogenous fungi. That this was probably not entirely the case is indicated by the observations of a number of men, including Holloway and Young (1943), who attributed the increase to the effect of the spray residues. Apparently a complex of several fluctuating factors is involved (see page 683). There

does appear to be a need, however, to redetermine the true pathogenicity of at least certain of the entomogenous fungi found on scale insects. That some of these fungi are actually saprophytes or secondary parasites is a distinct possibility. In fact, Fisher (1947) in her studies of certain of the entomogenous fungi (*Microcera*, *Podonectria*, and others) of scale insects in Florida makes the statement that "No evidence has been found that any of the so-called 'friendly fungi' actually parasitize any of the scale insects."

The fungi found on scale insects belong to a number of genera; and since many of them are important enough to deserve more than mere mention, it seems advisable to consider them according to their generic groupings, in much the same manner as that employed by Fawcett (1948). Some of the species discussed have at one time gone under other names; some have several synonyms which, for reasons of space, cannot be listed here.

***Sphaerostilbe* Infections.** Three important species of *Sphaerostilbe* are found associated with scale insects: *S. aurantiicola* (Berk. & Br.), *S. flammea* Tul. and *S. coccidophthora* (Zimm.). The first two species are found on insects in North and South America, the Orient, and in other parts of the world. *S. coccidophthora* occurs in the Orient on coffee-, tea-, and bamboo-scale insects. They are commonly called the "red-headed scale-fungi."

S. aurantiicola, which Snyder and Hansen (1945) have designated as *Nectria episphaeria* f. *coccophila* (Desm.) (= *S. coccophila* Tul.; *N. coccophila* (Tul.)), occurs on scale insects which live on citrus and other host plants. In North America, principally in Florida and the West Indies, the best known hosts on citrus include California red scale, Florida red scale, Spanish red scale, Putnam scale, ivy scale, snow scale, green scale, purple scale, thread scale, Glover scale, chaff scale, black scale, rufous scale, and San Jose scale. It has also been reported on the citrus mealybug. It appears to be one of the most prevalent fungi on scale insects, especially in Florida, where it is reported to have more "effect" on the purple than on the Florida red scale—the opposite of the pink fungus, *Nectria diploa* Berk. & Curt., which appears to be more "effective" against the red scale. The perithecia are small, globose, and orange red to blood red in color. The imperfect pustules are of the same color but are clavate or flattened pulvinate in form. The fungus has been obtained in culture from both conidia and ascospores. Working with the obscure scale, *Chrysomphalus obscurus* (Comst.), Luttrell (1944) observed that the fungus completely destroys the body of the infected scale and forms a plectenchymatous stroma between the shield of the scale and the bark of the plant host. The bark is not penetrated. On the other hand, Fisher

(1948) reports that all her attempts to artificially inoculate red and purple scales with the fungus have failed. (See also Fisher, Thompson, and Griffiths, 1948.)

S. flammea has been reported on the snow scale on citrus and on scale insects of many plant hosts besides citrus. The heads of the imperfect

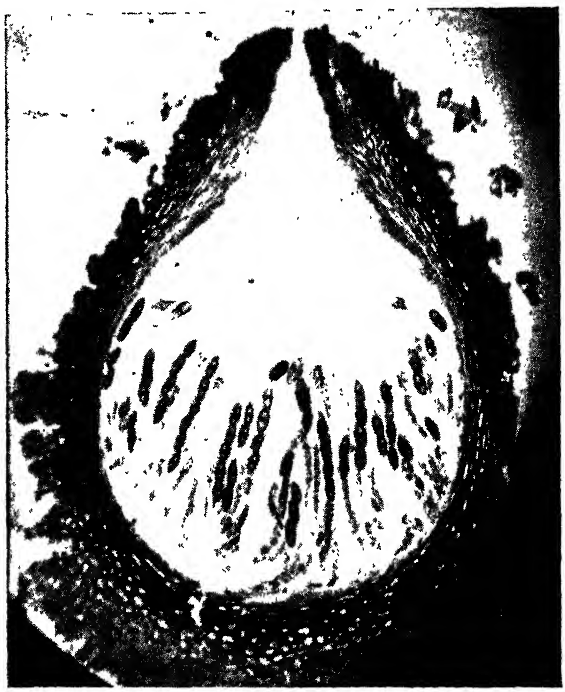


Fig. 112. Section of a mature perithecium of *Sphaerostilbe aurantiicola* (Berk. & Br.) (*Nectria episphaeria* f. *coccophila* (Desm.)), showing asci in all stages of development. (*From Luttrell, 1944.*)

form of this fungus are red in color; those of the perfect form are bright orange-red. The latter are crowded together on a stroma which often hides the scale insect.

The imperfect, or conidial, stages of *Sphaerostilbe* are generally placed under the generic designation *Microcera*, which, according to some authorities, is synonymous with *Fusarium*. Thus Snyder and Hansen (1945) have provided the combination *Fusarium episphaeria* f. *coccophila* for the imperfect stage of *Nectria episphaeria* f. *coccophila* (*S. aurantiicola*). Petch, however, still recognizes and uses *Microcera*.

***Nectria* Infections.** In the United States two species of *Nectria* are of importance: *N. diploa* Berk. & Curt., and *N. vilis* (Syd.). Concerning

the latter, only the imperfect form *Tubercularia coccicola* Stev. has been reported from North America, where it occurs on the snow scale, purple scale, and probably others. *N. barbata* Petch occurs on scale insects on citrus in Ceylon.

Nectria diploa, commonly known as the "pink fungus" in Florida, where it was discovered in 1912, forms pustules somewhat similar to those of *Sphaerostilbe*. The stroma is light rose or pink in color. A pink or reddish border surrounds the scale on which it has grown. It occurs on numerous scale insects in various parts of the world. It has its imperfect stage in *Microcerca* (or Petch's *Pseudomicrocera*). The Florida red scale has been reported to be particularly susceptible to its action, and Watson (1913) claimed success in spreading it artificially to good effect. The fungus also occurs on purple scale but with less frequency than on the red scale.

***Podonectria, Lisea,* and *Torrubiella* Infections.** In 1921 Petch proposed the generic name *Podonectria* (= *Berkelella*) to include three species of fungi commonly called the "white-headed scale-fungi": *P. coccicola* (E. & E.), *P. aurantii* (Hoeh.), and *P. echinata* Petch. All are found on scale insects.

P. coccicola was one of the first entomogenous fungi observed in Florida (Hubbard, 1885), where it occurs on Glover scale, purple scale, and chaff scale. It has also been found in South America, the Orient, and elsewhere. Watson and Berger (1937) are of the opinion that it was this fungus which saved the citrus industry of Florida in the 1830's when Glover scale (or the "long scale") was introduced into that state. The trees were at first killed back each year by the scale until presumably the fungus came to the rescue and saved them. Others feel that the fungus is in reality only a secondary parasite or a saprophyte which grows on the armors of the scales. Fisher (1947) observed it growing in such a location on purple scale that had been killed by the endoparasitic chytrid, *Myiophagus* sp. It usually appears as small whitish heads growing out from the scales. The imperfect form is *Tetracrium coccicolum* Hoeh.

P. aurantii is similar in appearance to *P. coccicola* and is found in Brazil on citrus and in Formosa on the black parlatoria scale. *P. echinata* occurs in Ceylon on *Lepidosaphes* sp.

Lisea parlatoria Zimm. has been observed on the black parlatoria scale on citrus leaves in Java. The perithecial heads are dark violet to black in color.

Torrubiella lecanii Johnst. occurs on the hemispherical scale in Cuba and Puerto Rico. Its perithecia are of a vivid yellow color. Incidentally, at least three species of *Torrubiella* have been reported from North America. One species, *T. gibellulae* Petch, a parasite of arachnids, is known to be

the perfect stage of *Gibellula aranearum* (Schw.). A considerable number of species originally placed in the genus *Torrubiella* are in reality members of other genera or synonyms of other species.

***Hypocrella* and *Aschersonia* Infections.** Members of the genus *Hypocrella*, included in the genus *Hypocrea* by early mycologists, have their imperfect stages in *Aschersonia*. The genus *Aschersonia*, as such, will be treated more fully in our section dealing with the fungi parasitic on whiteflies.

As has been pointed out by Petch (1921), even though species of *Hypocrella* so closely resemble the corresponding species of *Aschersonia* that it is not possible to decide which a given stroma is without sectioning it, yet it was apparently not until 1896 that any relation between the two was suggested. Since then it has definitely been ascertained that *Aschersonia* is the conidial form of *Hypocrella*. Nevertheless, the *Aschersonia* stage is found much more frequently than the *Hypocrella* stage. An *Aschersonia* stroma usually does not subsequently become perithecial although there are exceptions to this. In some gatherings all the stromata will be *Hypocrella*; in others all *Aschersonia*. Just what conditions govern the production of either stage is not known. Occasionally both stages may be found in the same stroma. It is by such findings that it is possible to correlate species of *Aschersonia* with their *Hypocrella* stages.

In 1921 Petch wrote that 70 species of *Hypocrella* and 60 species of *Aschersonia* have been described. Of these, however, only a total number of 54 names covering 42 species are valid. In the group found on scale insects there were then known to be 20 species of *Hypocrella*; the corresponding *Aschersonia* was known in 11 cases. In 1947 Petch informed the writer that because certain material was not available to him during World War I, these figures should actually have been slightly different. At any rate, in the scale-insect group of fungi, Petch states that there are now known to be 22 species of *Hypocrella*, the corresponding *Aschersonia* being known in 12 cases, and 3 unattached species of *Aschersonia*. Charles (1941*b*) lists 8 species of *Hypocrella* known in the United States, at least 5 of which have known *Aschersonia* stages.

Whereas species of *Sphaerostilbe* and *Nectria* occur on the armored scales (Diaspididae), species of *Aschersonia*, on the other hand, infect soft scales (Coccidae) and whiteflies (Aleyrodidae). In the latter case, there is also a difference between the two families in that those *Aschersonia* parasitic on whiteflies have paraphyses (elongated sterile cells) in the pycnidium, while those parasitic on the coccids have no paraphyses. It might further be mentioned that considerable care should be exercised when examining scale-infested trees to decide just which insect a *Hypocrella* is infecting. If an armored scale and a soft scale occur together on the same plant, the

fungus may destroy all the soft scales, leaving only the armored scale. Cursory examination may then lead the observer to think that the armored scales he sees were the hosts of the *Hypocrella.*

As far as scale insects are concerned, the better known *Hypocrella* and their *Aschersonia* counterparts include *Hypocrella epiphylla* (Mass.) (*Aschersonia cubensis* Berk. & Curt.), *H. turbinata* Berk. (*A. turbinata* Berk.), and *H. javanica* (P. & S.) (*A. coffeae* P. Henn.).

***Myriangium* Infections.** Although not a Hypocreales, but of the order Myriangiales, the genus *Myriangium* merits consideration at this point because it is one of the important parasites of scale insects throughout the tropics. Modern descriptions of the genus have been presented by Petch (1924, 1946) and by Miller (1938, 1940). In the words of the latter author, the plant body of *Myriangium* consists of a black pseudoparenchymatous stroma, which later gives rise to a peculiar type of apothecium with an apically delimited fertile region, consisting of asci at different levels embedded in coalesced fungous tissue. Apparently there is no conidial stage. The stroma is superficial on the bark of the tree, and under each stroma are several dead scales, penetrated and covered by mycelium. There is a definite relationship between the death of the scale and that of the limb and the *Myriangium.* Live stromata are not found on dead branches.

The commonly known species of *Myriangium* include *M. duriaei* Mont. & Berk. (*M. curtisii* Mont. & Berk.), *M. floridanum* Hoch., and *M. montagnei* Berk. Several authors have cited these species to be effective natural control agents, especially against citrus scales.

Other Fungous Parasites of Scale Insects. In addition to the species of Hypocreales and Myriangiales mentioned in the preceding paragraphs, a number of fungi parasitic on scale insects exist in other groups of fungi. Most of them remain among the Fungi Imperfecti.

Cephalosporium lecanii Zimm. occurs on several species of scale insects in the Americas and in certain islands of the South Pacific. In Florida it has been found on a number of scales on citrus and from a natural-control standpoint has been reported to be an effective parasite of the pyriform scale. In Brazil it is considered to be important in the control of the green scale on coffee plants, where it affects all the internal parts of the insect, including the eggs. The fungus grows around and over the scale insects. It is at first white but finally becomes pale yellow or lemon yellow in color. It usually has a powdery or mealy appearance, which is due to the numerous minute heads or spore clusters that develop on the conidiophores breaking out through the scale. Eventually it may appear smooth and waxy because of the fusion of the conidial heads. The fungus grows well on artificial media. Some authorities consider this fungus to

belong in the genus *Verticillium* along with another species, *V. cinnamomeum* Petch, which is found on scale insects and whiteflies in Florida. Other species of *Cephalosporium* have been reported on scales as well as other insects in countries other than the United States.

Similar to *Verticillium* is the genus *Cladobotyrum*, which contains at least one species, *C. heterocladum* (Penz.), parasitic on scale insects. It was described originally as *Verticillium* on the brown scale in Italy, and this or a closely related species has been so designated in Florida.

Several species of *Fusarium* have been reported on scale insects in various parts of the world. *Beauveria globulifera* (Speg.), the so-called "chinch-bug fungus," is sometimes found on the brown scale. *Spicaria javanica* Bally occurs on several scales in Ceylon as well as on the cottony cushion scale there and in Florida, where its control value has been noted when conditions were optimum. *Aspergillus depauperatus* Petch (considered to be a strain of the *A. restrictus* Smith series not far from *A. gracilis* Bainier) has been identified on specimens of California red scale on citrus in Palestine, and on other species in England and Ceylon. In certain parts of the world representatives of the genera *Pegiotrichum*, *Acrostalagmus*, and *Rhinotrichum* are found parasitizing scale insects. Species of *Cladosporium* are also found in this relationship but probably only as saprophytes or weak parasites.

In moist seasons along the California coast, a white powdery fungus appears on the black scale, sometimes killing considerable numbers of them. This fungus has been known as "*Isaria*" although no true *Isaria* stage has been observed. Quayle and Tylor (1915) were able to kill a fair percentage of scales with this fungus in moist chambers in the laboratory, but attempts to initiate outbreaks in the field were unsuccessful. Incidentally, as early as 1898 attempts were made to control the black scale in California by means of fungi, with favorable results being reported (Woodbridge, 1906).

Although on the whole the relationship between scale insects and species of *Septobasidium* (Basidiomycetes) is one of mutualistic symbiosis, enough of any colony of scale insects are parasitized to warrant the mention of the genus along with other fungi parasitic on this group of insects. The details of this relationship have already been discussed in Chap. 4.

Fungi Parasitic on Whiteflies

A considerable number of species of fungi are parasitic on the immature stages of several species of whiteflies (Aleyrodidae). Particular attention has been given to these combinations of parasitism in Florida, where the two most prominent species of whiteflies on citrus are the citrus whitefly, *Dialeurodes citri* (R. & H.), and the cloudy-winged whitefly, *Dialeurodes*

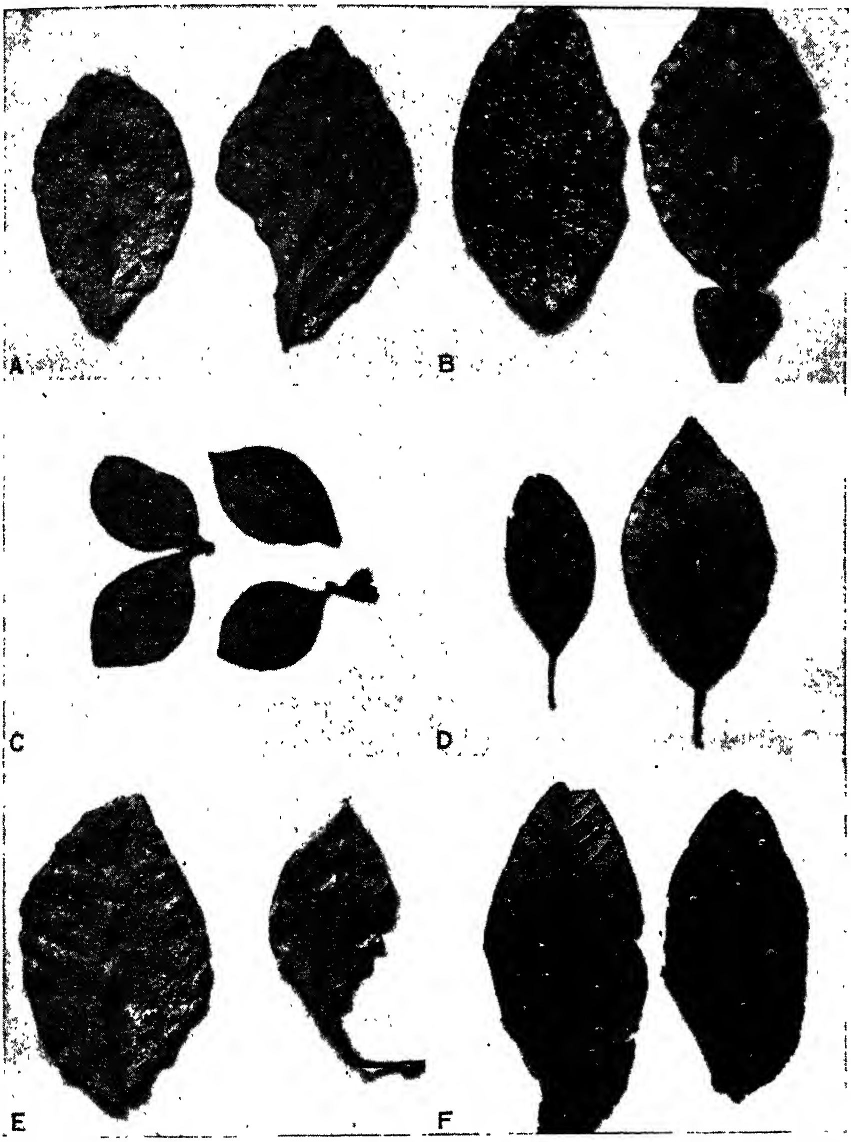


Fig. 113. Whiteflies and a scale insect bearing entomogenous fungi. *A. Aschersoni goldiana* Socc. & Ellis on the cloudy-winged whitefly, *Dialeurodes citrifolii* (Morg.). *B. Aschersonia aleyrodis* Webber on the cloudy-winged whitefly. *C.* Uninfected nymphs of the citrus whitefly, *Dialeurodes citri* (R. & H.), on leaves of Cape Jasmine (*Gardenia*). *D. Aegerita webberi* Faw. on the citrus whitefly. *E. Verticillium cinnamomeum* Petch on the citrus whitefly. Leaf to the right harbors uninfected whiteflies. *F. Aschersonia cubensis* Berk. & Curt. on the pyriform scale, *Protopulvinaria pyriformis* (Ckll.). (*Photographs by K. M. Hughes.*)

citrifolii (Morg.). At least five species of fungi occur commonly on the nymphs of these two species. These are of enough importance to merit separate discussions.

***Aschersonia aleyrodis* Webber.** The so-called "red aschersonia," *Aschersonia aleyrodis*, occurs on a number of species of whiteflies other than its principal hosts in this country. The fungus has its perfect stage in *Hypocrella libera* Syd. The imperfect stage forms a raised, flattened, pulvinate, red to pinkish-buff, stromatic pustule. It was first described in 1897 by Webber, who observed that, although all immature stages of the insect are readily attacked, those attacked while young were most abundant. In the area of Panasofkee, Florida, he noticed that the whitefly population decreased markedly with the spread of the fungus. In previous years the whiteflies and the accompanying sooty mold had been so abundant as to require the washing of all fruits. A few years after the fungus had extended its effectiveness no fruits had to be washed.

Soon after a nymph becomes infected with the fungus, it becomes swollen and may secrete more honeydew than usual. With the aid of a lens the intricately woven hyphae may be seen within the body of the insect. As the fungus develops internally, the interior organs appear to contract away from the margin. Soon after this the insect dies and the hyphae break through the body wall and form a dense marginal fringe around the edge of the insect. Subsequently the red pustule with its spores develops. As Fawcett (1944, 1948) has pointed out, what is often referred to by entomologists as "natural" or "unexplained mortality" may in reality be caused by the fungus in the early stages of its growth within the body of the insect. If conditions are such that the nymphs should become completely dried soon after death, the development of the fungus would be stopped before the latter became visible and in this way pass for "unexplained mortality."

The fungus was first cultivated by Fawcett (1907, 1908), using sweet-potato strips. Berger (1910, 1921) used this method in modified form for the production of large quantities of the fungus by inoculating sterilized sweet-potato slices in pint bottles. Matured cultures were obtained in this way in from 30 to 40 days and could be held in storage for extended periods of time (Watson, 1915). These bottles were then sent out to the growers who, after shaking the culture in water and filtering the suspension through cheesecloth, distributed the fungus artificially by spraying. One pint of culture was recommended for each acre of the orchard. The season in which the most successful results could be expected was during the rainy moist-weather period of June and July—Florida's rainy season.

The grower did not necessarily have to depend upon these cultures for a supply of the fungus. This could usually be secured by obtaining infested

leaves from another orchard. Such leaves, bearing the fungus-infected insects, could be placed in water and the resulting spore suspension used as a spray. Before the spray method was well understood, leaves bearing pustules of the fungus were pinned to trees infested with whiteflies. This method, though reasonably effective, was so slow that the spray method soon replaced it.

***Aschersonia goldiana* Sacs. & Ellis.** The yellow aschersonia, *A. goldiana*, for a number of years was identified in Florida as *A. flavocitrina* P. Henn. It has been reported on species of whiteflies in Florida, the West Indies, Panama, and on an unidentified insect in Venezuela. In Florida it is parasitic especially on the cloudy-winged whitefly, *Dialeurodes citrifolii* (Morg.), although it is occasionally found on other species.

A. goldiana resembles the red aschersonia in habits and general appearance, except that its pustules are yellowish in color. It can be grown on sweet-potato slices in much the same way as that used for the red aschersonia. It does not appear to be so effective a control agent as was first expected and is useful only in combating the cloudy-winged whitefly, for which it is probably no more effective than the red aschersonia. For interesting field reports on this and other entomogenous fungi, the reader is advised to consult the Annual Reports, as well as other publications of the Florida Agricultural Experiment Station during the early 1900's.

***Aegerita webberi* Faw.** This fungus, commonly called "Webber's brown fungus," occurs in the United States, the West Indies, Ceylon, India, and New Zealand. In Florida its principal hosts are the *Dialeurodes* species mentioned earlier and *Aleurothrixus howardi* (Quaint.). In Cuba the citrus blackfly, *Aleurocanthus woglumi* Ashby, is attacked. The fungus was first discovered in its sterile form in 1896 by Webber; its spore stage was found by Fawcett (1910*b*), who named it *Aegerita webberi*. It is a deuteromycete, the perfect form not having been found.

In its sterile form the mature fungus consists of a chocolate-brown compact stroma covering the entire insect, from the margin of which extend colorless thick-walled hyphae. Later in the development of the fungus, usually during the summer or fall, the marginal hyphae grow out long and colorless, extending not only over the undersurface of the leaf but also around the edges and upon its upper surface. The hyphae may sometimes extend down the petiole and along the stem to the next leaf. Every larva in the area covered becomes infected. When the hyphae become very abundant, the fungus may almost entirely cover the leaf surface with silky grayish-brown strands. The sporodochia, which consist of aggregations of conidialike, inflated, spherical cells, are borne on the upper surface of the leaf, on short lateral hyphae. These aggregations usually remain in union and function as a spore. Their light weight and

radiating appendages aid considerably in their dissemination by the wind.

The fact that *A. webberi* may spread by means of superficial hyphae spreading over the surface of the leaves, as well as by sporelike aggregations of cells carried by air currents or by insects, reportedly makes it one of the most efficient parasites of whitefly nymphs, provided that conditions for growth are favorable. Its ability to spread rapidly was one of the characteristics noticed by Webber when he first found this fungus in part of a 5-acre orchard in Manatee, Florida. At that time millions of live nymphs could be found in the trees of this orchard where the fungus had not spread. There was no trace of the fungus in adjoining orchards. Nine months later the fungus had spread so rapidly that it was difficult to find a living specimen of the whitefly in this 5-acre orchard. Trees in the adjoining orchards remained heavily infested with the insect. Within an additional year's time, the fungus had spread over a radius of approximately 2 miles, killing all immature stages of the insect as it spread. Although the accuracy of these observations has been questioned (Morrill and Back, 1912), Webber himself did not see fit to alter his opinion. That the sporodochia themselves might spread the fungus was shown experimentally by Fawcett (1910*b*) when he inoculated nymphs by drawing over them a camel's-hair brush moistened with water containing the aggregated sporelike cells. The insects showed signs of infection in 9 days, and in 16 days the stromata burst through the edges of the nymphs.

Since no satisfactory method of growing *A. webberi* in pure culture in large quantities has been developed, its distribution is usually effected by one of the three following methods: (1) pinning onto leaves containing whitefly larvae other leaves bearing infected insects and mature pustules; (2) washing off or triturating the pustules in water and spraying this suspension on the infested trees; (3) planting young fungus-bearing trees in such a way as to have their leaves intermingle with the whitefly-infested trees. In certain areas and under optimum conditions satisfactory control of whitefly larvae was obtained using these methods. Natural outbreaks, especially in certain low-lying hammock groves, are sometimes equally effective, making artificial remedial measures unnecessary.

Fusarium aleyrodis **Petch.** This fungus has already been referred to in connection with scale insects. It is best known, however, for its parasitization of whiteflies, *Dialeurodes citri* (R. & H.), and *D. citrifolii* (Morg.). Because it presents a delicate fringe of white hyphae growing outward from the edges of the larvae, *Fusarium aleyrodis* is commonly known as the "white-fringe fungus." It is probably the best known of about six identified and a considerable number of unidentified entomogenous species of *Fusarium* in North America.

The hyphae at first bear one-, two-, or three-celled conidia, oval to fusiform in shape. Later pinkish spore masses are formed on the edge of the larvae. The fungus is readily cultivable in pure culture.

The role of this fungus in the control of whiteflies in Florida does not appear to be so definite as is the case with some of the others. Under certain conditions, Watson (1913) found that 98 per cent of the larvae showing a "natural mortality" were infected with the fungus even though it was not apparent to the unaided eye. Adult whiteflies were also observed to harbor the fungus, which Watson succeeded in isolating from them. The eggs of the insects apparently also become parasitized and destroyed by the fungus.

Other Fungi Parasitic on Whiteflies. Similar in general appearance to the pustules of Webber's brown fungus are those of the cinnamon fungus, *Verticillium cinnamomeum* Petch, first seen in Florida in 1905. It has been found on both whiteflies and scale insects. Several species of *Aschersonia* and *Hypocrella* have been reported on aleyrodids in various parts of the world, including the West Indies and the Orient. According to Petch (1947), in the whitefly group of fungi, there are 13 species of *Hypocrella* of which the Aschersonia stages are known, and 14 unattached species of *Aschersonia*. Another Hypocreales, *Stereocrea aurantiaca* Petch, occurs on whiteflies in Florida and is the perfect stage of an *Aschersonia*. In Ceylon, *Rhinotrichum album* Petch has been observed on a species of aleyrodid.

An unidentified species of *Sporotrichum* has been isolated from the adult winged stage as well as from the nymphs of whiteflies in Florida, and it was thought to be responsible for the death of large numbers of them.

Other Ascomycete Infections

In the past some authors have considered many of the entomogenous fungi that we have discussed under Hypocreales as belonging to the order Sphaeriales. Modern treatments (*e.g.*, Wolf and Wolf, 1947), however, clearly distinguish between the two orders. Both are extremely large groups, but as now constituted, most of the entomogenous species fall in the order Hypocreales. Only a few are included in the Sphaeriales. The entomogenous members of some genera, *e.g.*, *Sphaeria*, have been placed in synonymy with certain Hypocreales, *e.g.*, *Ophicordyceps*.

As far as North America is concerned, the remaining orders of Ascomycetes contain relatively few species of fungi definitely pathogenic for insects. Species such as *Scorias spongiosa* (Schw.) in the order Dothideales have been found associated with insects but are probably saprophytic in nature, since the initial substratum frequently consists of insect secretions. A species of *Eurotium* (order Eurotiales) has been listed by Charles

(1941*b*) on a chalcid. Some primitive Eurotiales grow saprophytically on the pupal cases of certain Lepidoptera. Since this order includes many species of *Aspergillus* and *Penicillium,* ordinarily known in their imperfect or conidial stages, it is entirely possible that some of these entomogenous Fungi Imperfecti will eventually be grouped here. The genera *Cenangium* and *Sceleroderris* of the order Helotiales have at times been used to include entomogenous members, most of which have now been placed in synonymy with other Ascomycetes.

Although members of the large and important order Laboulbeniales may be considered as cutaneous parasites, they are generally thought of as commensals. For this reason they have been treated along with the nonpathogenic microbiota in Chap. 4 and will not be considered further here.

The Muscardine Diseases

Since the term "muscardine" has been indiscriminately used in entomological literature to mean almost any type of fungous infection, it appears desirable to consider the origin and proper use of this word.

As far as its modern usage is concerned, the term "muscardine" apparently originated in the Italian language with the word "*moscardino,*" meaning a musk comfit, grape, pear, and the like, or any of the various plants with musk-scented foliage or flowers. ("Musk," incidentally, refers to the odorous substance from the abdomen of the male musk deer, used as a basis for perfumes.) The French have the words "*muscadin,*" meaning a musk lozenge, and "*muscardin,*" which, in addition to referring to the dormouse (*Muscardinus*), also means a comfit or bonbon. Because the bodies of the insects infected with the fungi we are about to describe are transformed into white mummified specimens resembling in appearance comfits or bonbons, the natives of France referred to them as "*muscardin.*" French scientists added a final "e" to the word and used it in referring to the fungus concerned. It has since been taken over as a bona fide English word, and English dictionaries and encyclopedias furnish us with at least three meanings or uses of the word: (1) as a noun meaning the fungus; (2) as a noun meaning the disease caused by the fungus; (3) as an adjective, "muscardined."

The word "muscardine" was first used as it applied to the well-known disease of the silkworm, and also referred specifically to the fungus *Beauveria bassiana* (Bals.). Soon thereafter it was also used in reference to the fungus we now know as *Metarrhizium anisopliae* (Metch.), and because of the green color of the spores it was called "green muscardine." In addition to these two infections, it might be permissible to use the term generally in connection with those mycoses in which the fruiting bodies

arise on the exterior of the insect, producing a thick covering about the animal. The fungi concerned are known primarily in their imperfect stages (Fungi Imperfecti).

Muscardine of the Silkworm

Muscardine of the silkworm, *Bombyx mori* (Linn.), occurs throughout the world wherever this insect is reared. The disease has been particularly important in France and Italy, where tremendous losses have been experienced. Even after the nature of the muscardine and its control were understood, losses were considerable. For example, in northern Italy, around 1925, approximately 11 million pounds of cocoons were being lost every year from muscardine alone. Outbreaks still occur, but for the most part they are sporadic. In Italy, incidentally, the disease was first called "*mal del segno*" (the disease having a sign); later and at present it is known as "*calcino*" (calcium; white powder).

Early History. It is not strange that muscardine was the first acknowledged disease of the silkworm, since it is so easily recognized. The white mummies into which the diseased larvae are transformed are very distinctive and noticeable, and early sericulturists were able to ascertain their presence readily.

Until 1835 it was generally believed that the disease was not of a contagious nature but that it was caused by a variety of agencies, notably those pertaining to meteorological conditions and rearing techniques. For example, Boissier des Sauvages (1763) believed that it was due to a particular state of the atmosphere which precedes storms and which is called "*touffe*" (*i.e.*, "wisps of heat"). Nysten (1808) attributed the disease to defective incubation. Dandolo (1825) declared that it resulted from abnormal physiological conditions. The white efflorescence that develops on cadavers was, according to him, "the original mineral." Other causes were postulated by other writers, but most of them were variations of these amicrobic influences.

The credit for first showing that the disease is a contagious one and that it is parasitic in origin is generally given to Bassi de Lodi (1835–1839), who showed that the affliction was caused by a fungus that multiplied in and on the body of the silkworm. This discovery naturally created quite a sensation among sericulturists and microbiologists alike. Other investigators were drawn to this interesting case of parasitism. The fungus itself was studied and described by Balsamo, who gave it the name *Botrytis bassiana*, the specific name honoring Bassi. Audoin (1837*a,b*) elaborated on Bassi's discovery and demonstrated that this "cryptogam" would reproduce the disease when inoculated artificially into the body cavity or the fatty tissue of a healthy silkworm. This was true regardless of the

age or instar of the caterpillar. In order to ensure the completion of the fungus's development, however, certain humidity requirements were necessary. Audoin also observed that the malady was not peculiar to the silkworm but that many other insect species were also susceptible. In 1839 Johanys was able to cultivate the fungus on nonliving organic media.

The contagiousness of muscardine was not, however, accepted by all biologists and sericulturists without question. It took some years before the relation between the fungus and its host was thoroughly understood. Such a recognized authority as Guérin-Méneville (1848), for example, doubted the contagiousness of the infection and believed that the manifestations of the fungus were merely symptoms or signs of careless handling. Vittadini (1853), however, did a satisfactory job of refuting Guérin-Méneville's arguments and of showing that the fungus was, in fact, the true cause of muscardine.

Among the more modern workers (*i.e.*, since 1900) on this silkworm disease or on the fungus itself are included such names as Quajat, Verson, Conte and Levrat, Beauverie, Arnaud, Paillot, and Masera. These and others have contributed to our knowledge of muscardine until now it is a well-understood infection and one that can be coped with intelligently.

The Causative Fungus. As we have already mentioned, Balsamo placed the fungus responsible for muscardine in the genus *Botrytis* of which *Botrytis cinerea* Pers., the cause of a vine disease, is the type species. In 1911 Beauverie (1914) studied the fungus of muscardine, comparing it with yet another closely related species (*B. effusa*) found on silkworms, and he showed that the two species possessed common properties and that it was necessary to create a new group to include them. Shortly thereafter (1912) Vuillemin, who in 1910 was preoccupied with modifying the classification of Hyphomycetes of the Fungi Imperfecti, created the genus *Beauveria* of which the species *bassiana* became the type. Although Clements and Shear (1931) have since placed the genus *Beauveria* in synonymy with *Phymatotrichum*, most authorities retain the former name. Therefore, according to present usage, the name of the fungus responsible for muscardine of the silkworm is *Beauveria bassiana* (Balsamo) Vuillemin. Following the original taxonomic work on *B. bassiana*, a number of workers (*e.g.*, Dieuziede, 1925; Arnaud, 1927; Lefebvre, 1931*a*,*b*) made comparative studies of the several species in this genus, including *Beauveria effusa*, *B. densa* (Lk.) (= *B. tenella* Del.), and *B. globulifera* (Speg.), all of which are capable of infecting the silkworm. The distinct identity of the genus is now generally recognized.

The morphological and physiological characteristics of *Beauveria bassiana* have been studied in culture as well as in nature. The fungus

grows well, at an optimum temperature of approximately 28°C., on most of the artificial media used to cultivate fungi. It characteristically produces a flat, mealy, chalky, pulverulent growth, with spore formation taking place in from 3 to 7 days. When the spore is placed in water it becomes swollen in 24 to 48 hours and puts out one or more slender thin-walled germ tubes. About 32 hours later the germ tubes range from a few microns to about 80 microns in length, and the branches arising from these are very short. Conidial development begins at this stage, and later, when conidia are abundantly produced, they are borne in rather compact globose heads, either on the main hyphal branches or on short laterals that are usually at right angles to the main axis. This branching may be repeated, forming compact heads (Lefebvre, 1931*b*).

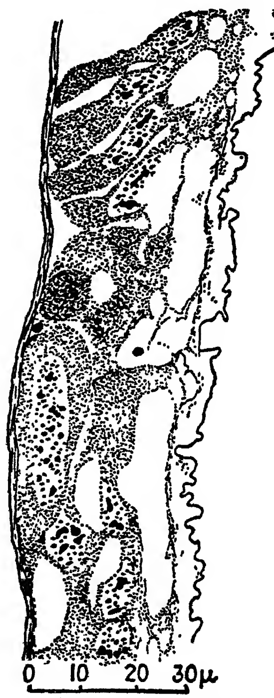


Fig. 114. Section of hypodermis of a silkworm infected with *Beauveria bassiana* (Bals.) showing region adjacent to the zone of penetration of the fungus into the insect. (*Redrawn from Paillot, 1930.*)

The Disease in the Silkworm. Infection of the insect by *Beauveria bassiana* begins soon after the animal's integument becomes contaminated with the spores of the fungus. Certain workers believe that the insect also becomes infected by way of the tracheal openings or the digestive tract. Infection by these routes, however, apparently occurs infrequently. It appears that in most instances the infection occurs by a direct penetration of the integument by the infecting germ tube. The germ tube is produced within about 2 days after the spore or conidium lands on the insect. Infection is facilitated by the presence of warm temperatures and humid atmosphere; although if these conditions are maintained for more than 24 hours, the rate of infection is usually retarded. As the mycelial filament penetrates the chitin the latter appears to be dissolved or digested. The filament penetrates through the cuticle and is met by an increased number of blood cells which become intermingled with the fungous threads. Phagocytosis may take place to some extent, but apparently it does not afford much real protection, since the fungus usually keeps on developing even at the expense of the blood cells.

During this invasion the hypodermis is destroyed in the area immediately surrounding the infecting hypha. Hypodermal cells adjacent to

this area also show pathological manifestations. For example, large vacuoles appear in the distal ends of the cells, *i.e.*, the end next to the newly forming cuticle (see Fig. 114). Furthermore these cells do not stain so deeply with acid fuchsin as do normal cells.

Once within the body cavity of the silkworm, the fungus continues its development in a characteristic manner. As early as 1853 Vittadini gave a fairly accurate description of this process. He showed that in the blood of the insect the fungus multiplies in the form of short filaments or hyphal bodies. The volume of the blood diminishes, and the blood cells are destroyed in proportion to the development of the disease. The physical-chemical properties of the blood change, as is indicated by the fact that the acidity diminishes and approaches neutrality. The formation of unidentified crystals in the blood has also been reported. As the infection proceeds, the circulation is slowed, then stops, and the consistency of the body becomes pasty. General paralysis sets in, followed by the death of the insect. According to Paillot (1930), invasion of most of the caterpillar's solid tissues, such as the fat body, does not occur until after the death of the insect.

After death the insect's body takes on a reddish tinge and becomes more and more hardened. The exact nature of this red coloration has been a subject for argument. Perroncito as well as Masera believe it to be due to the presence of the red-pigmented bacterium, *Serratia marcescens* Biz. Within 24 to 48 hours after death, the body is covered with a white network, which finally takes on a mealy aspect after the formation of the conidia. At the same time there appears a white inflorescence of an essentially crystalline nature. Verson gave the composition of this material as a double oxalate of magnesium and ammonium. Its true nature and identity have not been confirmed. A common post-mortem change seen in some insects infected with *B. bassiana* is the liquefaction of the internal tissues. This liquefaction is usually not followed by the formation of spores by the fungus.

That the lethal action of *B. bassiana* may not be entirely parasitic is indicated by Dresner's (1947) observation that a steam-acetone extraction of mycelium produces a substance that, even when greatly diluted, has a marked insecticidal action against certain mosquito larvae. He also noted that the germinating fungus secretes a chemical that possesses a knockdown effect on houseflies. Within half an hour after being dusted with spores of the fungus, the flies began dropping from the walls and ceiling of the moist chamber in which the tests were run. Within 3 hours there was 100 per cent knockdown with no subsequent recovery. A very high relative humidity appeared to be necessary to bring about the phenomenon.

Individual silkworms show very little true immunity against the

fungus. The fact that larvae of the fifth instar are more sensitive to infection than are those of the first instar does not in itself represent a true immunity in the latter but rather is the result of mechanical and physiological barriers. Apparently there has been a diminution in the severity of the disease since the early part of the nineteenth century. Some workers have postulated that this may be due to a decrease in the virulence of

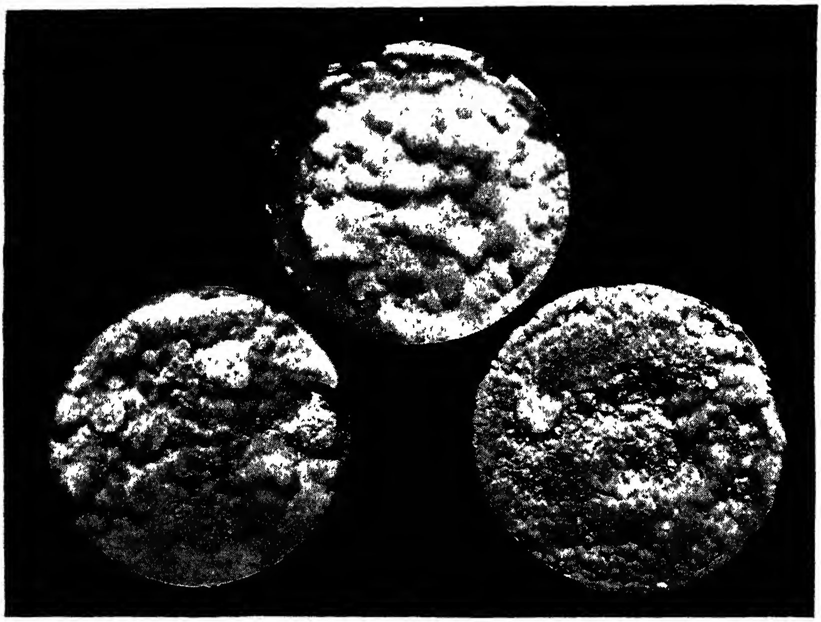


Fig. 115. *Beauveria bassiana* (Bals.) growing on vegetable media. Left to right, the substrata are ground corn, ground bush beans, and crumbled rat-ration pellets. The differences in sporulation are due to differences in humidity and not in media. (*Courtesy of E. Dresner, Boyce Thompson Institute, and Ohio State University.*)

the fungus, a developed immunity in the insects, or both. There is not proof that any of these occurs, however, and it is more likely that the decrease in incidence has been the result of better handling of the silkworms and the adoption of more sanitary precautions. Strains of *Beauveria bassiana* kept on artificial media have been known to retain their virulence for extremely long periods of time. Frequent transfer does lower its virulence somewhat, and it is therefore better to keep a single culture for a long period of time than to transfer it frequently if the maintenance of a high virulence is desired.

Transmission of Fungus. *Beauveria bassiana* is transmitted from one silkworm to another principally through contact and contamination. The fact that infection occurs by the cutaneous route enhances the effectiveness

of this mode of transmission. The fungus is not transmitted through the egg. Adult moths, however, may contract the disease, and, in fact, death may occur more rapidly among them than among the caterpillars. Although not of much consequence, transmission from one generation to the next may occur when conidia contaminate the exterior of the eggs and thus infect the larvae that hatch from them. Such contaminated eggs may be disinfected and the danger of infection thus removed.

The principal means by which the fungus is transmitted from one generation of insects to the next is made possible by the great longevity of the conidia or spores. These remain alive from one season to the next in the rearing rooms, and it is by such preservation that the disease is carried from generation to generation in most cases. Although in moist air the duration of vitality would be considerably shorter, it has been shown that the spores remain viable in dry air for at least 5 years.

Methods of Combating Muscardine of the Silkworm. Measures employed in the control of silkworm muscardine may be divided into two groups: those taken during the course of rearing and those taken between rearing seasons or without regard to these seasons.

When the disease strikes during the rearing season, the sick caterpillars should be removed and burned before the appearance of the conidia, which are formed about 48 hours after the death of the insect. The ailing larvae may be picked out by hand, but a more convenient method is that of using a wire mesh or perforated paper as a barrier. The healthy caterpillars have the ability and energy to crawl through the openings in this barrier to fresh food; the diseased ones remain behind and with the litter should be collected and burned.

Numerous attempts have been made to apply chemical and gaseous disinfectants without interfering with the vitality and development of the silkworms. Formaldehyde vapors and burning sulfur have been used in Europe with varying degrees of success. They are somewhat impractical because of the care necessary to maintain a dosage which is effective and yet which is not harmful to the insects. For example, the burning of 100 grams of sulfur per cubic meter is effective, but it also is injurious to the insects. Smaller dosage, 20 to 30 grams per 100 cubic meters, repeated daily for considerable periods has been recommended (Paillot, 1930). Similar difficulty has been had with the use of other compounds. Masera (1940) found "Procid," a mixture of copper oxychloride and hygroscopic substances, to be very effective against the fungus, but toxic against larvae that ate it. Similar results were obtained with sodium bisulfite.

Between rearing seasons, the equipment and surroundings should be thoroughly cleaned and disinfected. Almost any effective fungicide may

be used to destroy the fungi, as long as it is capable of destroying the resistant spores. For many years fumigation with sulfur in the presence of water vapor was used, especially where expense was a factor. Formaldehyde is perhaps one of the most effective vapors, since it is penetrating as well as fungicidal. Commercial formalin is useful in disinfecting the rearing equipment, as are numerous manufactured fungicides. A commonly recommended disinfectant is copper sulfate used in a 5 per cent solution. Some writers suggest a much lower dilution (*e.g.*, 1 pound of copper sulfate in 100 gallons of water). Pulverized copper sulfate powder added to walks and pathways leading to the nurseries is another precautionary measure.

In countries like France and Italy, where the disease has been particularly severe, laws and regulations have been established to aid in its control. When the disease makes its appearance in a silkworm nursery it must be reported to the proper officials. Quarantine placards are posted, and no one associated with a diseased colony is permitted to visit a healthy one.

The measures mentioned here for the control of silkworm muscardine may, to a considerable extent, be applied to most fungous diseases of other insects. Modern insectaries, as well as individual entomologists, are occasionally troubled by the occurrence of fungous diseases among stocks of insects being reared for experimental and other purposes. The experiences of sericulturists with muscardine provide information that may have general application.

Beauveria bassiana Infections in Other Insects

In North America, at least 30 species of insects have been reported as hosts of *Beauveria bassiana.* Numerous additional species have been cited from Europe and other parts of the world. Some of these insects represent serious agricultural pests, and their susceptibility to this fungus deserves further mention.

Infection in the Corn Borer. The European corn borer, *Pyrausta nubilalis* (Hbn.), is readily attacked and killed by *Beauveria bassiana.* This was shown with certainty when Metalnikov and Toumanoff (1928) showed that *B. bassiana,* as well as certain other fungi, was capable of causing disease and death in the corn borer when experimentally infected. Corn-borer larvae were much more sensitive to infection than were larvae of the wax moth, *Galleria mellonella* (Linn.), which was also susceptible. The first report of *B. bassiana* on the corn borer in North America was that by Lefebvre (1931*a,b*), who observed the mycosis among laboratory specimens at Arlington, Massachusetts, and imported from Manchuria. He was able to distribute the disease readily both in the laboratory and in the field. In 1934 Bartlett and Lefebvre reported on rather extensive field tests using the fungus against the corn borer in eastern United States

with encouraging results. A considerable reduction in the larval population was obtained after fields of infested corn and weeds were dusted with a mixture of spores and flour. Canadian workers (Stirrett, Beall, and Timonin, 1937; Beall, Stirrett, and Conners, 1939) then undertook similar field trials in Ontario, obtaining controls of 60 to 70 per cent. They emphasized the fact that the time of application of the fungus is of much greater importance than is the rate of application. A similar emphasis

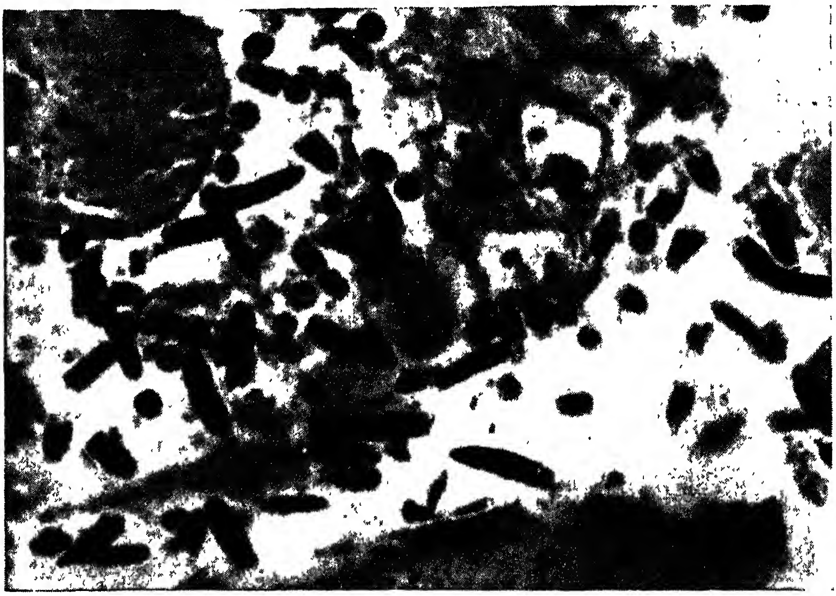


Fig. 116. Section of a European corn borer, *Pyrausta nubilalis* (Hbn.), infected with *Beauveria bassiana* (Bals.), showing hyphal bodies. (*Photograph by K. M. Hughes.*)

was made by Steyaert (1935) in the case of *Stephanoperes hampei* Ferr., a pest of coffee in the Belgian Congo. In this instance infection with *B. bassiana* must be effected before the insect has tunneled into the berry.

The pathogenesis of the infection in the corn borer has been worked out by Lefebvre (1934). The germinating spore on the surface of the larva produces an infection hypha, which penetrates the integument of the insect at any point except the head. This penetration appears to be assisted by the production, on the part of the hypha, of substances that dissolve the chitinous layer of the body wall and by mechanical pressure exerted by the fungus. Lefebvre asserts that he found definite indications that infection may also take place by way of the alimentary tract. After infection through either point of entry, the fat body is the first structure to be attacked. (The reader will recall that according to Paillot this tissue

was not invaded, in the case of the silkworm, until after the death of the insect.) The glandular structures and ganglia are next affected, while the last tissues to be attacked are the nervous system, the gonads, and the muscles. The fungus frequently penetrates the tracheae, greatly impairing the process of respiration.

The external appearance of a diseased corn borer is similar to that of a silkworm infected with the same fungus. Such a larva first becomes sluggish in movement, fails to respond to most external stimuli, and in most instances turns a characteristic pink color. The larva remains soft and pliable until the mycelium has ramified and grown throughout the various parts of its body. Following this the body becomes rigid and mummified. It can now be readily broken, and the whitened chalky contents can be crumbled into a powder. No external signs of the fungus are evident as long as the mummified larva is kept in a dry atmosphere. Soon after exposure to moist air, however, the white mycelium becomes apparent over the surface of the insect. Within a day or two conidia are produced abundantly giving the insect a mealy, powdery appearance.

Certain phases in the course of infection taken by entomogenous fungi in the corn borer have also been given attention by Toumanoff (1928, 1933), who worked not only with *Beauveria bassiana* but also with *B. globulifera* (Speg.), *Aspergillus flavus* Link, and *Spicaria farinosa* (Fron.). Larvae infected with either of the last two species move slowly or make but slight convulsive movements when touched. Small black spots appear on the skin; filaments of the fungus are found under these spots in a kind of abscess under the destroyed hypodermis. The filaments ramify into the body wall and into the abdominal cavity, eventually destroying all the tissues.

Infections in Other Insects. A number of other economically important insects are known to be susceptible to *Beauveria bassiana*. For example, Jaynes and Marucci (1947) found considerable numbers of codling-moth larvae, *Carpocapsa pomonella* (Linn.) dead of a *B. bassiana* infection in New Jersey apple orchards. Laboratory tests showed the fungus to be highly pathogenic when spores of the organism were either dusted upon or inoculated into healthy hibernating or freshly spun-up summer larvae. In the field, artificial dissemination of spores on the foliage brought about a significant increase in mortality. Spore dust or spray applied to infested fruit prevented the newly hatched larvae from making a successful entrance and also killed the larvae after entry. To a certain degree, therefore, the fungus is an agent of natural control on all stages of the larval population. It appears to be particularly important in cool wet seasons. Infected codling-moth larvae are characterized by the dark-brown lesions over the epidermis and a weakened condition of the insect, which becomes typically

mummified after the penetrating hyphae have consumed and displaced the body tissues. The fungus is capable of killing the larvae even when the moisture content of the air is low, but the external mycelial growth with its consequent sporulation is not produced unless considerable amounts of moisture are present. The fungi within the mummified larvae will remain viable for months and give rise to the white mycelial growth when moisture is supplied. Jaynes and Marucci assert that in a few infectivity tests larvae showing only a few lesions were able to recover. Since such recovery is rarely seen in insects, an investigation into its nature appears warranted.

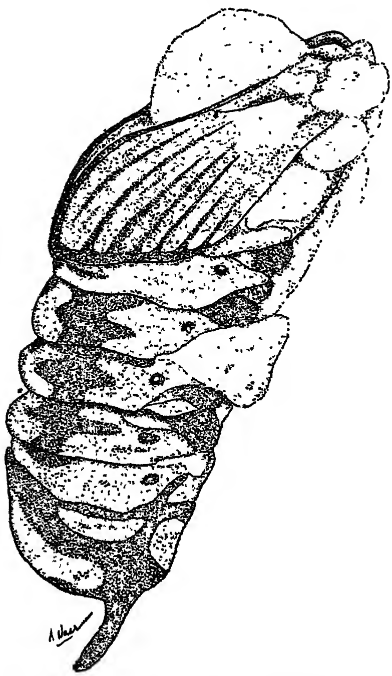


Fig. 117. Pupa of the California oak moth, *Phryganidia californica* Pack., infected with *Beauveria bassiana* (Bals.). The fungus may be seen breaking through intersegmental areas.

Although not observed in large-scale epizootics, *B. bassiana* has been found pathogenic for such insect pests as the Japanese beetle and the Colorado potato beetle. The latter insect is host to another *Beauveria*, *B. doryphorae* Poi. & Pat., in Europe where a number of important pests, such as *Pieris brassicae* (Linn.), are hosts to *B. bassiana*. The course of the infection in *Pieris* has been described by Arnaud (1927). Other susceptible insects are shown in Fig. 119.

White Muscardine of the Chinch Bug

Although at least six species of fungi have been reported as parasitic on the chinch bug, *Blissus leucopterus* (Say), only two have been found to be of significance as far as actual epizootics are concerned: *Beauveria globulifera* (Speg.) and *Empusa aphidis* Hoff. The latter is also a common entomophthoraceous parasite of aphids. It is frequently called the "gray fungus" to differentiate it from *Beauveria globulifera*, the "white fungus."

The chinch bug, one of the most injurious insect pests of cereal crops in the United States, was first noticed in 1783 in North Carolina. In 1840 it was reported in Illinois, and it has been under observation ever since

throughout the central Mississippi Valley states, rendering its greatest damage in the Corn Belt.

Apparently the first observer to record the presence of a white fungus on the chinch bug was Shimer, in 1865, at Mount Carroll, Illinois. Although the identity of the fungus was not determined, Shimer credited it with causing the destruction of vast numbers of the insect, especially in low, creek-bottom land during moist warm weather. His belief that it was a contagious disease did not gain ready acceptance by most entomologists, and several prominent ones (*e.g.*, Walsh and Riley) scoffed at the idea. Some years later, in 1882, Forbes, in Illinois, and Popenoe, in Kansas, confirmed Shimer's observations when they reported the susceptibility of the chinch bug to fungous attack. The fungus seen by Forbes and Popenoe was the so-called "gray fungus," *Empusa aphidis* (Hoff.).

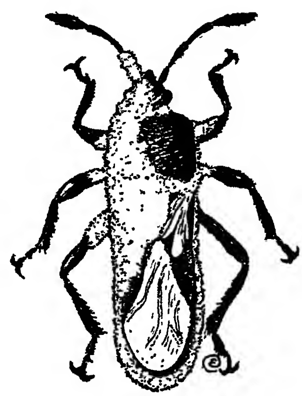


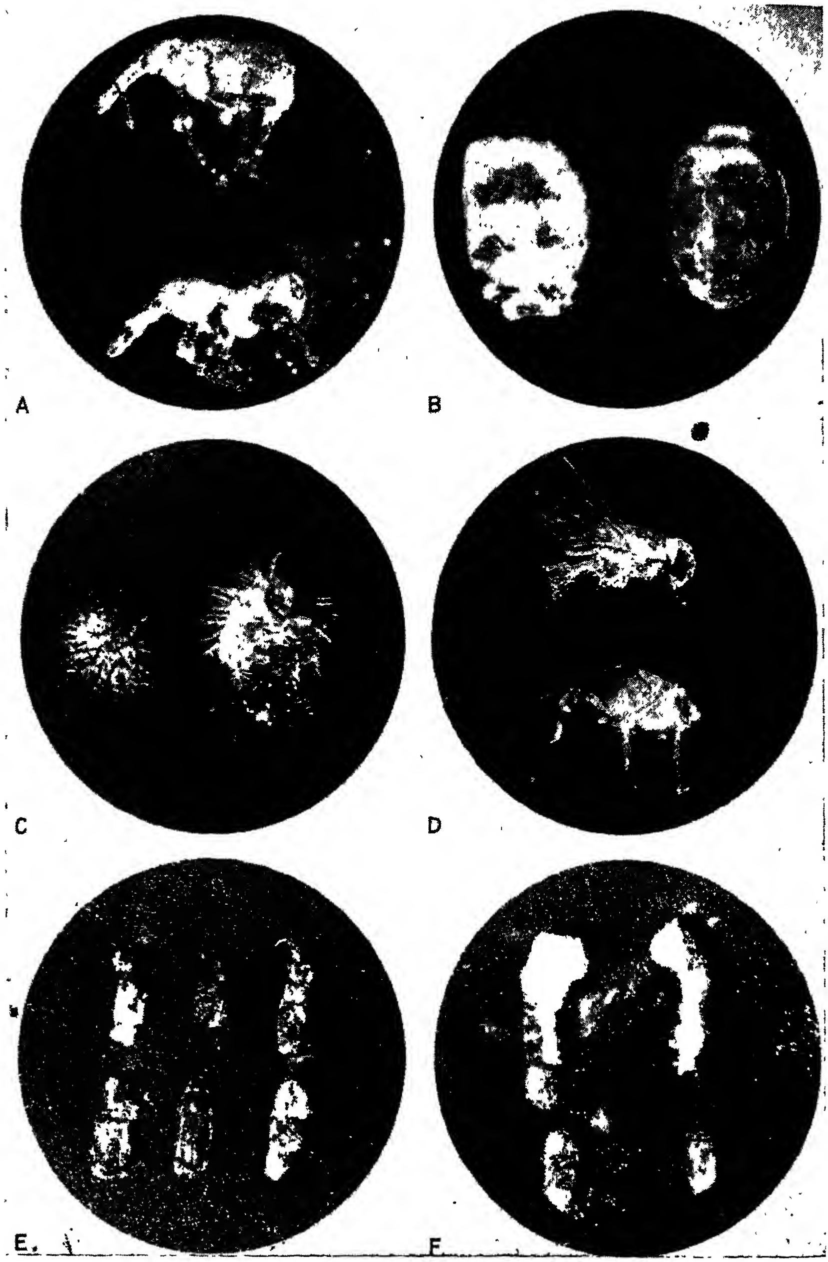
Fig. 118. Chinch bug, *Blissus leucopterus* (Say) killed by *Beauveria globulifera* (Speg.).

The white fungus, *Beauveria globulifera* (Speg.), was observed on chinch bugs by Forbes in 1887, in Clinton County, Illinois. Shortly thereafter it was reported from Minnesota, Iowa, Ohio, and Kansas. Thus more than 100 years elapsed between the time the chinch bug was discovered and the time the insect was found infected with the white fungus.

The Fungus. *Beauveria globulifera* (Speg.) Pic. (= *Sporotrichum globuliferum* Speg.), first described in 1880 from a wireworm adult from Argentina, has been reported on approximately 75 species of insects in the United States (see Charles, 1941*b*, for host list), and on numerous additional species in other parts of the world. Its parasitism does not appear to be so specialized as is that of many entomogenous fungi, since its hosts are in numerous families and in several orders of insects. It also grows on corn and certain other plants but not so well as on insects.

On its insect host, *B. globulifera* usually appears as a loose white cottony or mealy growth, at times almost completely enveloping the insect. At short irregular intervals the conidiophores bear minute heads, sessile in attachment and creamy-white in color, composed of conidia closely packed into a nearly spherical form. Some of the characters of this fungus on artificial media have been described by Pettit (1895), Lefebvre (1931*b*), and others. They are essentially the same as those of *B. bassiana* (Bals.).

Since temperature, humidity, and possibly light are variable factors that are able to cause marked changes in the activity of the fungus, a con-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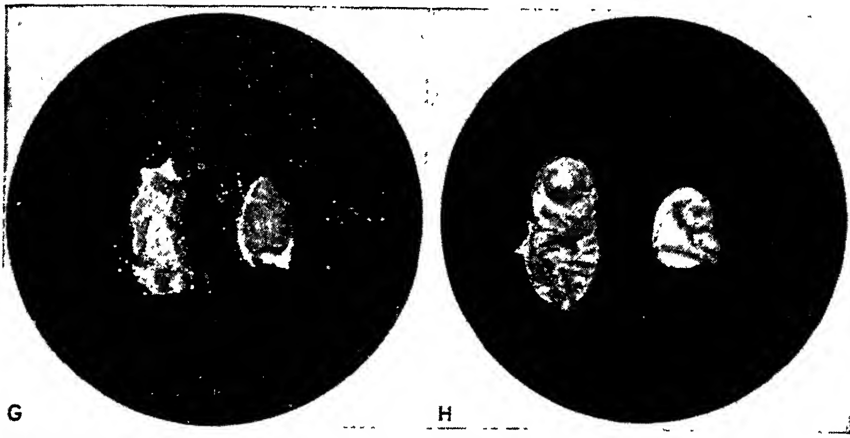


G H

Fig. 119. Various insects attacked by *Beauveria bassiana* (Bals.), (see pp. 379–380) showing formation of the white spores. *A*. Rose curculio, *Rhynchites bicolor* (Fabr.). *B*. Mexican bean beetle, *Epilachna varivestis* Muls. *C*. Larvae of the Mexican bean beetle. *D*. Housefly, *Musca domestica* Linn. *E*. Top, rice weevil, *Sitophilus oryza* (Linn.); bottom, confused flour beetle, *Tribolium confusum* Duv. *F*. Black carpet beetle, *Attagenus piceus* (Oliv.). Top, larvae; bottom, adults. *G*. Bean weevil, *Acanthoscelides obtectus* (Say). *H*. American cockroach, *Periplaneta americana* (Linn.). (*Courtesy of E. Dresner, Boyce Thompson Institute, and Ohio State University.*)

sideration of their effects is warranted. Such a study was made by Headlee and McColloch in 1913; and, because their data might in a general way apply to other muscardine fungi, it is quoted from their publication:

TEMPERATURE. Field temperatures may influence the fungus in two ways—the extremes may cripple and destroy it, and the optimum encourage and further its growth.

Careful laboratory studies at this station have shown that the exposure of spores of the fungus to 104°F. to 105°F. in a saturated atmosphere for twenty-four hours does not prevent strong growth, that an exposure for forty-eight hours destroys most of the spores, and that an exposure for seventy hours does not kill them all. Spores allowed to develop for forty-eight hours, and then exposed to this temperature and moisture, perish. Spores previous to germination, in a comparatively dry atmosphere, may be exposed for five hours to as high as 209°F. without injuring the germination.

Spores, dry or wet, may be exposed over night to low temperature, even when the mercury reaches as low as −18°F., without apparent injury. Spores freshly sown were exposed to changing temperatures from January 5 to February 12, during which time the temperature changed from freezing to thawing, or *vice versa*, twenty-one times—the daily mean was below 32°F. for nine days, and the minimum temperature 15°F.—without injury. Growing fungus threads subjected to repeated freezing and thawing temperatures are not destroyed.

While these facts render it quite possible that the fungus growth, encouraged by moist, cool weather in exposed and cultivated fields, might be destroyed by succeeding hot weather, for the exposed dusty soil will reach about 135°F. at the surface, it is very unlikely that the ordinary foci of the disease, sheltered as they are beneath heavy growth of weeds and grass, would be even seriously injured, for in such location the temperature rarely or never reaches 91°F. It is not likely that the fungus, even in cultivated fields, would be eradicated by dry, hot weather, because while exposed mycelium would perish, the dry spores can withstand far higher temperatures than they would experience, and in the process of cultivation much of the fungus must be thrown beneath the surface of the ground, where the temperature rarely exceeds 100°F.

In view of the fact that the maximum air temperature of the last twenty-five-year period at Manhattan has been 113°F. and the minimum −35°F., it does not seem probable that extremes of temperature such as the fungus is likely to experience in Kansas will ever seriously reduce, not to say eradicate it.

On bee-broth agar the fungus makes good growth at 70°F. and 80°F., less vigorous growth at 90°F., and no growth at all at 50°F. and 100°F. The growth comes a little more quickly at 80°F. than at 70°F. The optimum temperature for its growth and spore production on this medium is therefore between 70°F. and 80°F., probably about 75°F.

As might be expected, the temperature for best growth on chinch bugs is the temperature for best growth on this medium. Only three temperatures were given careful trial—50°F., 70°F., and 90°F. The bugs succumbed to the disease most readily in 70°F.

In general, the average mean temperature in this state [Kansas] is such that the fungus can grow well from April to October, while it is more or less completely dormant from October to April.

Moisture. Moisture of two sorts appears to influence the growth of the chinch-bug fungus—relative humidity and water.

The fungus will not grow in a relative humidity of 90 per cent or less, but will remain dormant for an indefinite period. We have kept it in dried corn-meal culture for more than eighteen months, and found it perfectly virile at the end of that time; found it would grow readily and would destroy chinch bugs. It is hardly conceivable that the chinch-bug fungus could have too much moisture in a state of nature. Dashing and washing rains might carry much of it away, but would serve merely to distribute it, and enough would in all probability be left to carry the disease on. The best degree of moisture for germination seems to be a film of water, although germination will take place in a relative humidity of a little less than 100 per cent.

These facts indicate that while the fungus can not grow in dry weather, it is unlikely to be eradicated by any extreme of moisture it is likely to experience in Kansas. As a matter of fact, this agrees with universal experience with this fungus. It thrives in wet weather, disappears during dry periods, and springs up again on the advent of sufficient moisture.

Light. General germination of the spores in a film of water required under a temperature of 75°F., twelve days, while subjected to normal daylight coming

through north windows, and when in complete darkness germination became general in one day less. In a confirmatory experiment general germination was made simultaneously in thirteen days. Ordinary daylight is thus shown not to be seriously hostile. This also is borne out by field experience.

The Infection in Chinch Bugs. The course of the infection with *Beauveria globulifera* in the chinch bug is very much like that which takes place with similar fungi in other insects. Under very humid conditions, the conidium lying on the integument of the chinch bug germinates by sending out a tube that penetrates the body wall of the insect. The bug dies of the infection in about 3 days. Hyphae fill the body cavity of the

Fig. 120. Grasshopper infected with *Beauveria globulifera* (Speg.) showing the fungus emerging through the thin intersegmental areas of the exoskeleton. (*Drawn from photograph by Marchionatto, 1934.*)

bug, finally penetrating to the outside, where the body is covered with the typical white mycelial growth. Numerous conidia are formed on the conidiophores, and when these land on other insects the cycle is repeated if conditions of temperature and humidity are favorable. As might be expected, the disease spreads most rapidly when the bugs are crowded together or are very numerous on the host plants.

According to Smith (1933), the fungus also kills chinch bugs, beetles, and other insects during the winter and in the early spring while they are still in hibernating quarters, in grass clumps, under stones, boards, and in wheat fields. The fungus thus can overwinter in protected situations that supply the proper moisture conditions, and it is carried to the field by the spring migration of the chinch bugs.

As demonstrated by Snow (1896), healthy chinch bugs may be infected with *B. globulifera* experimentally. This may be accomplished simply by dusting the insects with the fungous spores. Chinch bugs of all ages are attacked, but the older the bug the more easily it succumbs. Bugs that have been weakened or injured from any cause appear to be more susceptible to attack than are those which are in perfect condition. Adults of the overwintering second brood are more resistant to the fungus than are the adults of the first brood.

Use as Control Agent. The efforts of early workers to use *B. globulifera*

in the control of the chinch bug constitute an interesting and important phase in the history of microbial control. The failure of the fungus to fulfill the high hopes of those who advocated its use serves as a warning to be remembered whenever excessive enthusiasm is expressed over the control value of newly discovered insect pathogens, especially when this control depends upon the artificial distribution of the microorganism.

The first attempt to bring about an outbreak of the disease by artificial dissemination was that by Lugger, in 1888, who distributed diseased bugs in several localities in Minnesota. Although the experiment appeared successful, Lugger suspected that, since the disease spread so rapidly, the spores of the fungus were already present in the test fields and that he had only reintroduced them.

In 1888 F. H. Snow began his observation and experiments in Kansas; these extended through 1896. In 1891 the Kansas state legislature established an experiment station at the University of Kansas to propagate the fungus and to distribute it free of charge. It was placed under Snow's direction. Almost 50,000 packages of the fungus were distributed by this organization, but the true value of the program was never ascertained with certainty. The reports of observers in 1891 and 1892 were very favorable, whereas those during succeeding years were less favorable. The probability that the fungus was widely distributed naturally could not be ruled out.

Distribution programs were carried on in states other than Kansas, but in each case the work was eventually dropped. Lugger in Minnesota tried it again in 1895 but abandoned the method by 1902. It was similarly abandoned in Illinois, Nebraska, Missouri, Ohio, and Oklahoma. The artificial distribution of the fungus did not appear to affect materially the incidence of the disease in chinch-bug populations or the effectiveness of the control brought about by the infection.

An appraisal of the effectiveness of these distribution programs was made easier by the work of Billings and Glenn, who, in 1911, published the results of their comprehensive observations on the disease. The following points were among those set forth in their conclusions:

1. The chinch-bug fungus is present naturally in fields everywhere throughout the infested area in Kansas.

2. It is present in such great abundance that any artificial distribution of infection in a field would be too insignificant, by comparison, to be of practical use.

3. Its distribution naturally through a field is much more uniform than any artificial distribution can be made.

4. The amount of fungus used experimentally in both wheat and corn fields

was so far in excess of any that would be used by the farmer in infecting his own fields that he could not reasonably expect to succeed.

5. The fungus shows little tendency to spread from centers of artificial infection. The apparent rapid spread of the fungus is due to favorable conditions bringing it into activity simultaneously over considerable stretches of territory.

6. In fields where the natural presence of the fungus is plainly evident its effect on the bugs cannot be accelerated to any appreciable degree by the artificial introduction of spores.

7. In fields where the fungus is not in evidence spores introduced artificially have no measurable effect.

8. Apparent absence of fungus among chinch bugs in a field is evidence of unfavorable conditions rather than lack of fungus spores.

9. All the benefits of the Sporotrichum [Beauveria] disease of chinch bugs may be realized by merely letting the fungus naturally present in the soil do the work of extermination as far as it will.

10. Moisture conditions have much to do with the appearance of chinch-bug disease in a field; artificial infection nothing.

11. Spent adult chinch bugs succumb to attack more readily than younger ones, but as the old bugs have finished depositing their eggs, their loss by fungous disease accomplishes little else than increasing the amount of the infectious material.

12. Laboratory experiments can be made to prove that artificial infection accomplishes results upon bugs confined in cramped quarters and without food, but in the field, where fresh and usually drier air prevails and food is abundant, an entirely different situation is presented.

13. Advocating artificial infection or encouraging it by sending out diseased chinch bugs does not serve the best interests of the farmer, since his attention is thus diverted from other and more efficient methods of combating the pests.

14. The reported success of former years on the part of farmers is believed to be due to the following causes: (1) failure to recognize spontaneous outbreaks of the disease because of previous artificial sowing of infection, and also failure to use check, or untreated fields as a basis of comparison, thus claiming the outbreak as directly due to artificial infection; (2) failure to distinguish the skins of molted bugs from dead bugs; (3) mistaking the scattering of chinch bugs in cornfields for evidence of their death by fungous disease when carcasses were not present as proof.

Perhaps one of the most significant conclusions arrived at by Billings and Glenn was that in fields where the fungus was naturally present, its effect on the bugs could not be accelerated appreciably by the artificial introduction of spores. Fawcett (1944) has accounted for this fact by the suggestion that the wind-borne spores are produced in such great profusion, providing wide distribution in a short time, that the saturation point for infection is readily attained and that artificial distribution is therefore of little account. It should be remembered, however, that the

conclusions of Billings and Glenn apply particularly to the chinch bug, to *B. globulifera*, and to the localities in which they worked. It would be unsafe to make sweeping generalizations regarding all insect mycoses solely on the basis of their findings.

What may be accepted as the most general currently accepted opinion with regard to the control value of the fungus against the chinch bug has been expressed by Packard and Benton (1937). These authorities state

Fig. 121. Specimens of the sugar-cane-borer beetle, *Rhabdocnemis obscura* (Boisd.), infected with the green-muscardine fungus, *Metarrhizium anisopliae* (Metch.), and showing characteristic spore masses. (*From Speare, 1912; courtesy of J. P. Martin.*)

that *B. globulifera* is probably the most destructive natural enemy of the chinch bug; that it is generally present in fields throughout the country but that its effectiveness depends upon the weather; and that, since it has been proved that the spores of the fungus are present wherever the bugs are common, its artificial dissemination as a control measure is needless.

Green Muscardine

Upon examining containers of soil in which had been placed some larvae of the wheat cockchafer, *Anisoplia austriaca* Hbst., Metchnikoff, in 1879, observed some of the specimens to be dead. Two days after the insects died a white fungous growth appeared on their bodies, being particularly noticeable around the spiracles. This growth soon covered the animals entirely except the head. Later it took on a green color, eventually becoming a dark blackish-green. In the blood of the insects oval fungous bodies could be seen, which, as the infection progressed, filled the body cavity as a mycelial mass. For this fungus Metchnikoff first proposed the name *Entomophthora anisopliae*. Later, at the suggestion

of the botanist Cienkowsky, he called it *Isaria destructor*. Delacroix (1893) published on the fungus under the name *Oöspora destructor*. A few other synonyms (*e.g.*, *Metarrhizium cicadinum* v. Höh.) are to be found in the literature. Although one of the Fungi Imperfecti and not an entomophthoraceous fungus, the original specific name remains valid, and the organism is now known as *Metarrhizium anisopliae* (Metch.) Sor., as it was designated by Sorokin (1879). It is commonly referred to as the "green-muscardine fungus," being characterized by the dark-green color of its conidia, or spores. The green color, however, does not appear to be characteristic of all species of the genus, since another species, *Metarrhizium album* Petch, parasitic on leafhoppers in Ceylon, is white in color, and *Metarrhizium brunneum* Petch, parasitic on a cicadellid in the Philippine Islands, is yellow to brown. On the other hand, a fourth species, *Metarrhizium glutinosum* Pope, isolated from deteriorated cotton, has dusky olive green to olivaceous black conidia.

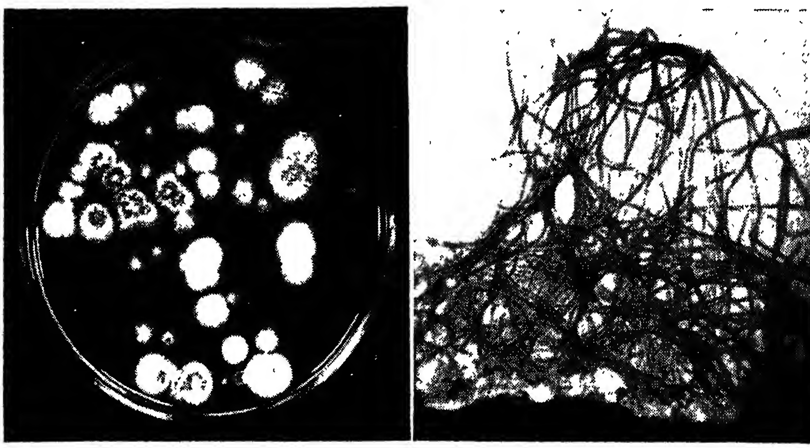
Metchnikoff's 1879 report is of particular significance, since in an addendum to his article he describes how he experimentally initiated the disease in healthy larvae. It was this experiment that may be credited with establishing the idea of intentionally causing a disease in insect pests of economic importance. Metchnikoff mixed spores of the fungus with the soil and placed the healthy larvae therein. After 10 days, 8 out of 9 larvae were dead of the fungus. In 1880 he reported a similar experiment in which larvae and adults of the sugar-beet curculio, *Cleonus punctiventris* Germ., exposed to spores of the fungus died within 12 days.

Since Metchnikoff's early observations a large number of additional insect species have been reported as hosts of the green-muscardine fungus. This includes approximately 70 species in North America, with probably an equal or greater number from other parts of the world. Apparently the first record of the fungus in the United States is that of Pettit (1895), who found it parasitic on the wheat wireworm, *Agriotes mancus* (Say) in New York State.

Characteristics of the Fungus. The green-muscardine fungus *Metarrhizium anisopliae* is usually considered to have a systematic position near *Penicillium* in the family Moniliaceae. Its perfect stage is not known, and the report that it is a *Cordyceps* is generally discounted. Pettit (1895) describes a variety *americana* and Johnson (1915) records forma *major* and forma *minor* occurring on different hosts. Most of the variations that have been noticed are concerned with differences in spore sizes and in coloration.

Metarrhizium anisopliae grows readily on artificial media and develops particularly well on potato media. Ordinarily it is easiest to start a culture by using the spores of the fungus. With proper techniques, however,

cultures may be initiated with nonfruiting forms. Upon germination the spore swells somewhat and a germ tube appears at one end and sometimes at both ends of the spore. As described by Speare (1912), this germ tube elongates, and branching occurs after about 30 hours and continues for several days. Certain of the branches extend upward and become the sporophores. The latter are usually closely packed with their branches intertwined. After about a week they begin to form spores which are at first white, but gradually, as they mature, appear olive green in color.



A *B*

Fig. 122. *Metarrhizium anisopliae* (Metch.). *A*. Colonies of the fungus as they appear after 8 days of growth on potato agar. Fruiting and nonfruiting areas may be seen. *B*. Portion of the mycelium from a 5-day-old potato culture, showing the segmented hyphae. (*From Glaser, 1926.*)

The spores become partly cut off from the grouped sporophores and cohere in prismatic masses. Spore formation occurs by the simple abstriction of elongated buds which appear at the distal ends of the branched sporophores. Other buds form below the original one, and this continues until chains of spores are formed. So closely do the chains of spores arising from adjacent sporophores cohere that in old cultures they may form a crust over the surface of the medium and may be removed in large flakes. The sporophores are short and are branched in a manner somewhat resembling the branching in *Penicillium*, although the long unbranched basal portion of the sporophores usually associated with this genus is not seen in *Metarrhizium*. The spores may vary considerably in size but are usually within a range of from 5 to 7.5 microns in length to 2.3 to 3.7 microns in width.

Although only a meager amount of information is available relative

to the physiological properties of *M. anisopliae*, it may be assumed that they are in general similar to those of the white-muscardine fungi already described. High humidities and warm temperatures promote the growth and development of the fungus. In culture, a temperature between 24 and 26°C. appears to be optimum, the entire range for normal development being between 10 and 30°C. The ability of the spores to germinate is destroyed at temperatures between 55 and 60°C. for 5 minutes. The range of hydrogen-ion concentrations for the normal growth of the fungus is from pH 4.7 to 10. The optimum value lies between pH 6.9 and 7.4.

Fig. 123. A group of silkworms dead of infection by the green-muscardine fungus, *Metarrhizium anisopliae* (Metch.). (*From Glaser, 1926.*)

Although a medium containing organic matter is more favorable for the growth of *M. anisopliae* than is one having an inorganic nitrogen source, both types of medium can support its growth. No special requirements are necessary as far as the organism's carbon source is concerned. The growth and fructification of the fungus are retarded by rays of the sun. The spores may be kept in a dry condition for 3 years or more.

The Infection in Insects. From the knowledge already gained during studies on white muscardine of the silkworm, a general idea of the course of such infections in insects was at hand from the time *Metarrhizium anisopliae* was discovered. Not until 1926, however, was much attention paid to the details of the pathogenesis of *M. anisopliae* infections in insects.

In that year Glaser reported on his studies of the green-muscardine disease in silkworms. This was followed 3 years later by similarly detailed reports, by other authors, of the infection as it occurs in the European corn borer.

The silkworm, *Bombyx mori* (Linn.), acquires the infection via penetration of the integument by the germinating hypha. It does not appear to be susceptible via the digestive tract. After the fungus enters the body cavity it develops in the blood and does not penetrate the tissues until

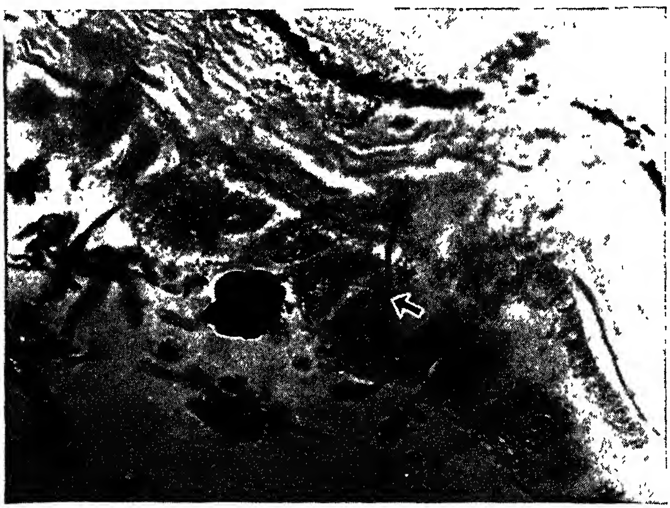


Fig. 124. Section through a silkworm that died 7 days after experimental infection with *Metarrhizium anisopliae* (Metch.). A hyphal thread may be seen penetrating through the hypodermis of the insect. (*From Glaser, 1926.*)

after the death of the insect. A reaction on the part of the host occurs, as is indicated by large masses of phagocytes that congregate around and between the fungous threads. The phagocytes are unable to offer very much protection, however, since the parasite is too large for ingestion. Four or five days after infection the larvae begin to lose their appetites, assume a yellowish color, and appear sick. In 5 or 6 days they become extremely feeble and die, after which they become rigid in form and brownish-yellow in color. The fungus now invades nearly all body tissues.

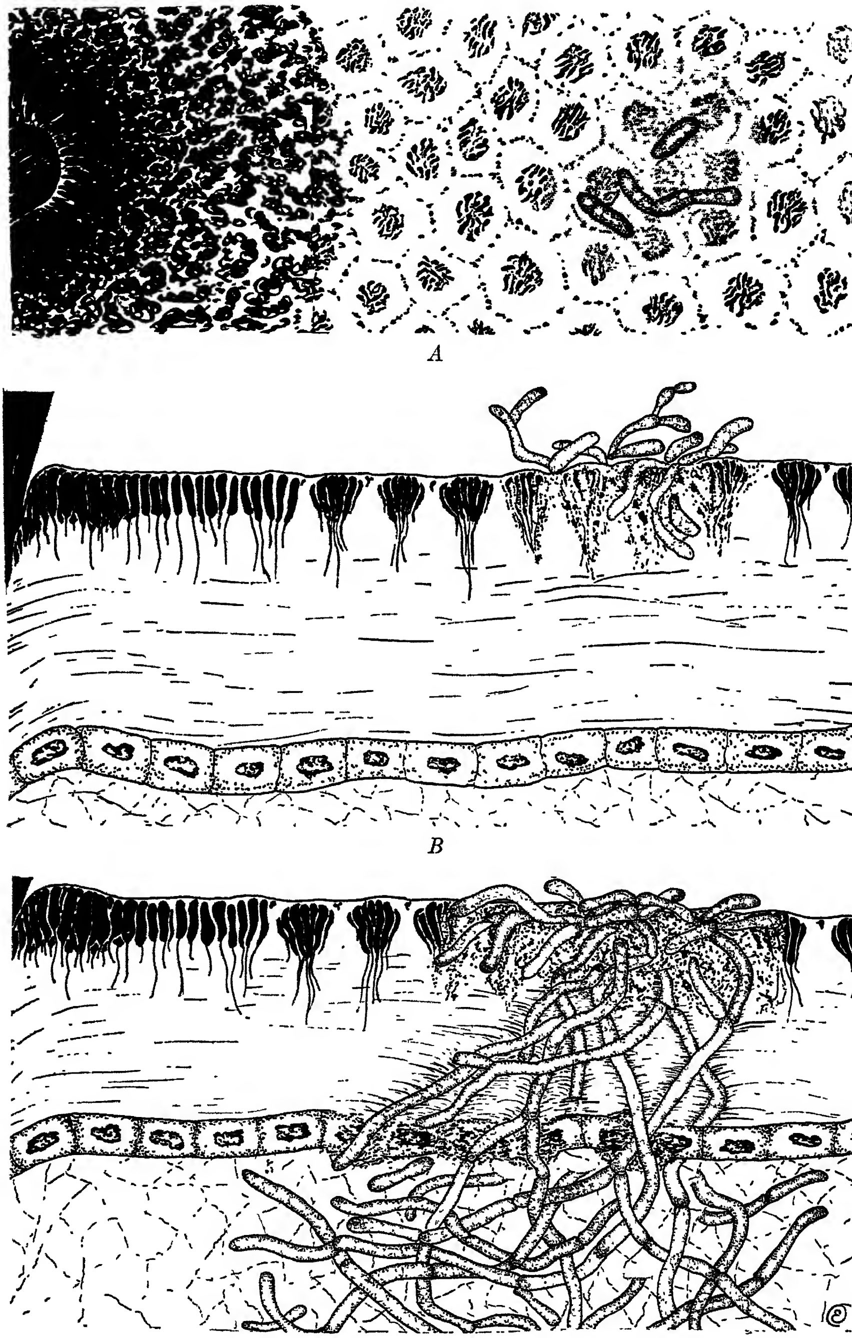
After the silkworm dies, certain of the hyphae penetrate outward, and 7 or 8 days after the beginning of the infection the hyphae break through the integument everywhere. The larva soon becomes covered with a white down consisting of hyphae destined shortly to become the sporophores. A day or two later the rather short and branched sporophores begin to form spores. These soon cover almost the entire body of the silkworm, and as they mature, the spores become olive green in color,

cohering in masses which may be removed in large flakes. The silkworm usually dies in the larval stage. Sometimes, especially among those infected during the last instar, some individuals will spin a cocoon and succumb within. In such cases the white down appears, but the fungus rarely fruits.

In 1929 Wallengren and Johansson published a description of the manner in which *M. anisopliae* infects the European corn borer, *Pyrausta nubilalis* (Hbn.), and in so doing added considerably to the information supplied by Glaser in connection with his silkworm studies. Infection of the corn borer takes place without any great difficulty; the conidia fasten very easily to the intersegmental folds and from there penetrate the integument. The intersegmental folds have a thinner chitinous layer than the other parts of the segments and therefore offer a more favorable place for infection. The germinating hyphae, however, can pierce the fairly thick integument on the dorsal side of the segments.

Upon examining under the microscope a piece of integument or skin dissected from a corn-borer larva, one can see that the areas immediately surrounding the bases of the hairs are darker and brownish and present a granular appearance produced by a number of small, round, dark pigment bodies, gathered close to each other immediately under the surface of the chitin. The rest of the skin has similar granules farther apart (Fig. 125*A*). Histologically, the body wall shows a lamellose structure, the lamellae lying parallel to the surface. The black pigmentary bodies lie immediately under the outer heavily sclerotized surface membrane. From each pigment granule emanate one or more threads, clustered together, which continue down into the cuticle to about a third of its thickness. The threads converge so that the entire formation resembles an inverted pyramid (see Fig. 125*B*). In the areas around the bases of the hairs, however, the threads are not gathered in pyramids but run straight down into the cuticle; they appear to be somewhat shorter than those in the pyramids. With this histological picture in mind, let us now consider the details of infection as conceived by Wallengren and Johansson.

With the proper conditions of temperature and humidity, the fungous spores, or conidia, fastened on the surface of the integument send out germinating hyphae. At those places where the conidia germinate the cuticle assumes a yellowish color. Under this yellow spot the pigmentary bodies become clotted and fall to pieces, and the threads disintegrate. Each hypha pierces the cuticle and begins its entrance into the body wall at the point of one of the thread pyramids, which disintegrates and becomes granular. The outer layer of the cuticle is more or less destroyed, and the area concerned assumes a more yellowish or dark-brown color. These colored spots increase in size as the infection progresses. It is



A

B

C

possible for the infection to occur without causing the brown spots. Also, after the hypha has penetrated the skin, the fungus appears to be able to continue its growth without further discoloring of the integument.

The growing conidial hyphae apparently secrete some sort of chitin-dissolving substance, possibly an enzyme, which opens or facilitates the way for penetration. The hyphae also exert a rather great pressure on the substratum. In sections, the cuticular lamellae can be seen to be pressed down when the hyphae are developing at right angles to it (Fig. 125*C*). The hyphae often grow for relatively long distances between the lamellae, evidently because growth in this direction offers the least resistance, but sooner or later they turn inward, perforate the endocuticula and hypodermis, and enter the body cavity. According to Wallengren and Johansson, the hyphae seem to have a special preference for the adipose tissue, but they also invade the muscular and central nervous systems. It is not entirely clear, however, whether these workers refer to premortal or postmortal conditions. Since their description at this point apparently refers to living insects, this observation is in conflict with Glaser's assertion that in the silkworm the fungus does not enter the internal tissues until after the death of the insect. In any case, after the corn borer dies, rapid growth of the fungus takes place, filling the interior of the body. Hyphae force their way through the body wall and appear as a white fluff on the surface of the integument. Finally, the characteristically green conidia are formed. Conidia are also formed within the body of the insect.

The symptomatology of the mycosis in the corn borer is similar to that which characterizes the infection in most larvae. The first symptoms include the appearance of yellow or brown spots on the cuticle; these usually spread over other parts of the body. The fungus seems to have some toxic influence on the host. Marked nervous disturbances become evident in the later stages of the disease. The larvae lose their appetites, become apathetic, make no spontaneous movements, and their irritability decreases more and more. Finally the insects lose their righting reflex, and death soon follows. The body finally becomes stiff and mummified. Under conditions of an atmosphere saturated with moisture and a temper-

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Fig. 125. (*On facing page.*) Diagrammatic representation of the manner in which *Metarrhizium anisopliae* (Metch.) infects the European corn borer, *Pyrausta nubilalis* (Hbn.), according to the studies of Wallengren and Johansson (1929). For a detailed description see text. *A*. Appearance of magnified portion of larval integument with base of hair at left; germinating fungous spores at right. *B*. Cross section of same area of the integument somewhat later, showing pyramidal pigment areas by germinating hyphae. *C*. Further penetration of the hyphae through the integument and hypodermis, and into the underlying fat tissue.

ature of about 25°C., death occurs in approximately 4 days. Occasionally an infected larva, under conditions not entirely optimum, will survive for a considerably longer period of time. It is unknown for larvae to survive longer than 26 days, however, once the fungus has penetrated the body wall. In corn-borer larvae the mycosis produced by *M. anisopliae* under experimental conditions has a death rate of 100 per cent.

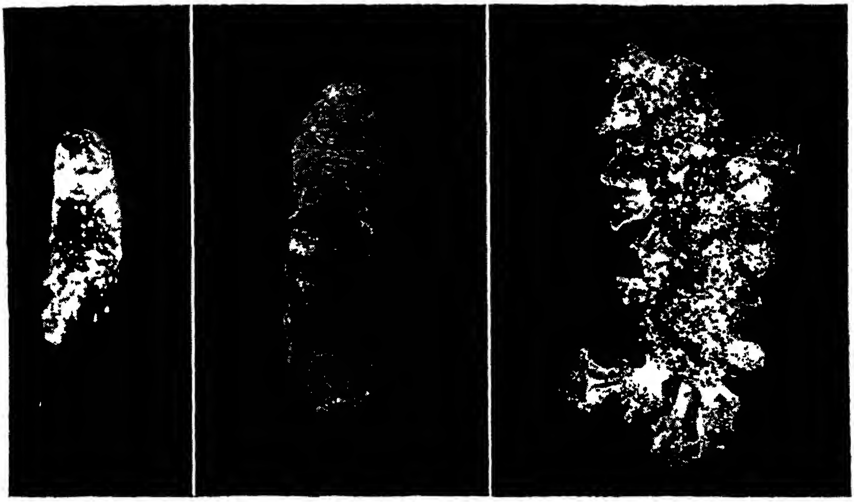
Fig. 126. The European earwig, *Forficula auricularia* Linn., killed by infection with *Metarrhizium anisopliae* (Metch.). (*From Crumb et al., 1941; courtesy of B. J. Landis, U.S. Department of Agriculture.*)

Use as Control Agent. Following Metchnikoff's (1879, 1880) discovery that the wheat cockchafer, *Anisoplia austriaca* Hbst., and the sugar-beet curculio, *Cleonus punctiventris* Germ., could be artificially infected with *M. anisopliae*, other workers soon considered using the fungus as a control agent on a wide scale. In southern Russia, entomological commissions were established in Kharkov and Odessa to investigate the practicability of artificially distributing the fungus to combat these insects, particularly the cockchafer. In 1886 and 1888, Krassilstschik undertook to use the fungus for combating the sugar-beet curculio, reporting field mortalities of from 50 to 80 per cent. In his laboratory at Smela, near Kiev, he produced spores of the fungus in large enough quantities to make field distribution practical, although still on a rather limited scale.

Further attempts to use *M. anisopliae* against various insect pests were made during the early years of the twentieth century in such parts of the world as Java, Hawaii, Trinidad, and Puerto Rico. In Trinidad, for example, Rorer (1910, 1913) used the fungus against the froghopper, *Tomaspis varia*, a pest of sugar cane, with apparently favorable results. The spores were usually distributed in the form of dusts (mixture of flour and spores) applied to the sugar-cane plants at the rate of 2 or 3 pounds to the acre. Specially constructed large culture cabinets were devised to produce the spore material in great quantities.

In some areas, as in Java (Groenewege, 1916; and Rutgers, 1916) and in Puerto Rico (Stevenson, 1918), the fungus, although an efficient parasite of several noxious insects, did not prove to be an entirely practical means

of control. Since it was rather widely distributed in nature, and since its effectiveness depended upon the existence of favorable climatic conditions, many workers decided that attempts to distribute the fungus artificially would be useless. In some localities excellent results were obtained after initial distributions, with a declining effectiveness upon subsequent distributions. The reasons for this are not clear, except that the initial



A B C

Fig. 127. Larvae of a cutworm, *Feltia gladiaria* Morr., infected with *Metarrhizium anisopliae* (Metch.). *A*. Mycelium before the development of spores. *B*. Larva covered with mycelium bearing mature spores. *C*. Crust of spores broken into small portions which are borne outward by the developing mycelium. (*From Crumb, 1929; courtesy of U.S. Department of Agriculture.*)

epizootics might have indicated the absence of or the rather low number of naturally existing spores in the area concerned.

In 1913, Friederichs reported excellent results with the fungus when directed against the rhinoceros beetle, *Oryctes rhinoceros* Linn., a pest of coconut. Infected beetles were placed in trap piles of rotten coconut husks and other debris, placed here and there throughout the coconut groves. The beetles gathered in these piles for egg laying. When the larvae hatched, nearly all of them were apparently attacked and killed by the fungus. According to Simmons (1939), however, "no efficient control seems to have come from the introduction" of the fungus into Samoa, although grubs are frequently found killed by the organism, and some officials maintain that it is still a beneficial agency. Simmons points out that regular provision of infected breeding traps is required and that

this necessitates constant careful supervision or the results are likely to be the reverse of what is desired.

In Europe a number of attempts were made to use the green-muscardine fungus against the European corn borer with rather encouraging but not convincing results. Hergula (1931), for example, found that, when infested corn plants were dusted experimentally with fungus-spore mixtures, the infestation was reduced to such a point that the damage to the plant by the insect was practically negligible. Data on practical large-scale field application were not gathered.

From the reports and information available, it appears that the value of *M. anisopliae* as a control agent is largely in the realm of natural control. The introduction of the fungus into areas, localities, or islands where it does not occur may be entirely worth while. Once the organism is established, however, it appears that, with certain exceptions, the artificial distribution of spores is useless. If and when favorable weather conditions prevail, the fungus, if present, exercises practically its full effectiveness without the help of man. There seems to be little reason for doubting that the sporadic and small but continuous occurrence of the disease among certain insects has its beneficial effect, even though this effect may rarely be enough to bring the insects under control from an economic standpoint.

Sorosporella Infection

Although the infection we are about to consider—one caused by *Sorosporella uvella* (Krass.) Gd.—is sometimes referred to as the "red muscardine," this terminology is not applicable in the same sense in which we have been using it in the foregoing discussions. Unlike the true muscardines, the fruiting bodies of the fungus in this infection are not as a rule produced as a covering on the exterior of the insects; and, instead of becoming mummified and hardened, the insects disintegrate soon after death.

One of the most thorough studies of the fungus and the infection it causes has been performed by Speare (1917, 1920*a*). Much of the discussion to follow will be based upon reports of this mycoentomologist.

The Discovery and Systematic Position of the Fungus. The fungus under consideration was first observed in Russia by Krassilstschik in 1886. He observed it in the same insect, *Cleonus punctiventris* Germ., on which he did so much work with the green-muscardine fungus, *Metarrhizium anisopliae* (Metch.). Krassilstschik named the newly discovered fungus *Tarichium uvella.* Two years later Sorokin (1888), another Russian scientist, found a fungous parasite of the cutworm *Euxoa segetum* (Schiff.), which he named *Sorosporella agrotidis.* In 1889 Giard pointed out the identity of *Tarichium uvella* and *Sorosporella agrotidis.* Since the genus

Tarichium was at that time employed for species of *Empusa* and *Entomophthora* in which only resting spores were known, and since the resting spores of the fungus under consideration were unlike those of any known *Empusa* or *Entomophthora,* Giard proposed that the generic name *Sorosporella* be used together with the specific name *uvella.* Giard's proposal has been generally accepted, even though this worker adhered to the belief that it was an Entomophthorales. That the fungus does not belong to this order was shown by Speare (1917, 1920*a*), who considered it a verticilliacious Hyphomycete (Moniliales; Fungi Imperfecti). This remains the systematic position generally accorded it today.

Probable synonyms include *Acremonium cleoni* Wize (1905) and *Fusarium acremoniopsis* Vincens (1915). *Massospora štaritizii* Bresadola (1892) appears almost certainly to be a *Sorosporella,* but since several of its characters are quite different from those of *S. uvella,* it is perhaps another species of this genus.

Symptoms and Pathology. Noctuid larvae parasitized by *Sorosporella uvella* exhibit no symptoms of disease during the first few days after being infected. Three or four days before death, however, they lose their appetites and become sluggish in movement. In some host species a change in outward appearance may be observed 1 or 2 days before death. Such larvae may turn a creamy-white color. Sometimes red-colored patches appear a few hours before death, either posteriorly, anteriorly, or more commonly near the middle of the body.

Soon after death, which usually occurs within 7 to 10 days after infection, the creamy-white color changes to pink. The pink color appears simultaneously over the entire body, becoming more and more intense until the final development of the fungus is reached and the insect is of a characteristic brick-red color. The body of the insect appears shrunken and wrinkled and somewhat flattened. A longitudinal, ventral, furrowlike depression is nearly always present in its abdomen. The cadaver is soft and pliable, and if a portion of the integument is indented, it remains sunken with little or no reaction. Unlike the case in many other fungous infections, the body is never hard and sclerotiumlike. No fluid exudes if it is pricked with a needle. If the body is torn open entirely, the internal fungus mass is seen to be coherent, of a shiny creamy or pink color, and of a gelatinous consistency.

Later the host's body becomes more shrunken and the reddish color more intense. The body wall is now rather brittle, the slightest shock serving to rupture it. The spores of the fungus become brick red in color, dry, dustlike, and less coherent than previously. Virtually nothing remains of the host's internal organs, and the body appears as a small sac filled with dust.

The histopathology of the disease has been studied to some extent by Speare (1920*a*) in connection with his observations on the yeastlike cells or "blastocysts," which represent the vegetative stage of *S. uvella*. These blastocysts multiply in the hemolymph by yeastlike germination until enormous numbers of them are floating in the circulating blood. They are found throughout the body cavity, within the heart, and wherever it is possible for the blood to penetrate. The hemolymph becomes so loaded

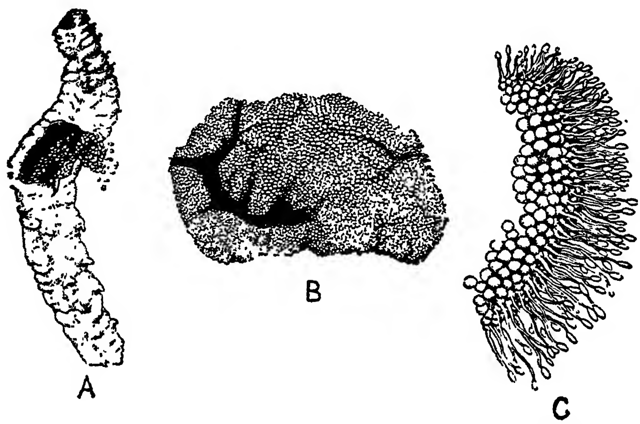


Fig. 128. *Sorosporella uvella* (Krass.). *A*. An infected cutworm torn open, exposing the resting-spore aggregations. *B*. A single resting-spore aggregation. *C*. A portion of a resting-spore aggregation germinating in water, and showing promycelial-like germination and conidia. (*From Speare, 1920a.*)

with blastocysts that not only is the circulation impeded but its life-sustaining elements seem to become depleted.

The blastocysts apparently never actually invade or penetrate into the living tissues or organs of the host. There appears to be a substance secreted by the fungus which causes the host's tissues to break down. The softer tissues disintegrate first, usually beginning with the fat body. A colony of fungus cells will multiply rapidly near the fat cells; and, after the wall of the fat body has broken down at the surface, the colony will tend to enter and take the form of the organ. As a result, lamellalike or vermiform convoluted colonies are formed. After the fat body, the muscles, nerve fibers, malphighian tubes, alimentary tract, and all the hypodermal tissues are gradually destroyed, until only fragments of the tracheae can be seen. The chitinous body wall, originally very thick, becomes very thin—apparently because of the action of a solvent of some kind.

Phagocytosis of the blastocysts is known to occur. This may consist simply of the engulfment of the parasites within the cytoplasm of individual phagocytic leucocytes, or it may take the form of phagocytic

complexes within which the blastocysts are included; these complexes usually consist of masses of phagocytes arranged in concentric layers about a number of blastocysts. The number of blastocysts contained within a single phagocyte varies from 1 to 15. When large numbers have been phagocytosed, the leucocytes are gradually destroyed. From the information at hand it would appear that the phenomenon of phagocytosis offers little, if any, real protection against the fungus once the infection is under way in a susceptible host. When a small number of *Sorosporella* conidia are inoculated into the body cavity of a relatively nonsusceptible host, such as the silkworm, something occurs, not known to be phagocytosis, which renders the fungus cells innocuous and causes them to disappear.

Life History of Fungus. When the blood of a cutworm infected with *Sorosporella uvella* is examined, 6 or 7 days after inoculation, the yeastlike cells, or blastocysts, will be seen floating free in the hemolymph. These blastocysts are the early vegetative stages in the development of the fungus. When young, the cells are elliptical in form, are hyaline, measure 8 by 5 microns in size, and in blood smears occur singly or coherent in pairs, rarely in threes. They multiply by a yeastlike germination; budlike outgrowths appear that grow rapidly until they reach the approximate size and form of their parents, then break away and proceed to form new cells by the same process. Multiplication continues until large numbers of coherent blastocysts are present in the form of colonies throughout the body cavity of the insect. Small colonies coalesce so that large masses are formed which ultimately develop into resting-spore masses. When these spore masses mature they eventually appear typically as brick-red "dust" within the cadaver of the host insect. It is in this condition that specimens of the diseased insects are most often collected in the field. The resting spores, or chlamydospores, mark the termination of the development of the fungus.

In order to determine the origin, nature, and function of the resting spores, Speare (1920*a*) conducted a number of experiments dealing with the organism's life history. Under the stimulus of moisture and suitable temperature, the protoplasm of the resting spores swells, producing budlike protuberances which soon assume the shape of a germ tube, branch freely, and become septate. The fully developed conidiophores are supplied with bottle-shaped branchlets or sterigmata at the tips of which are borne the conidia, which are quite unlike the resting spores from which they arose. The conidia are elliptical in form, thin-walled, vacuolate at each pole, and measure 4 to 6 by 9 to 11 microns in size. The development and structure of the conidiophores and conidia are typical of the verticillate Moniliales, of which the genus *Sorosporella* is a member.

Germination of the resting spores in unbroken larvae may be induced by

placing the insects in circumstances that provide the proper moisture and temperature requirements. In a few days an external fungous growth on the unbroken integument of the host becomes visible. The germina ions thus obtained are confined only to those spores which lie near the surface, immediately beneath the integument. When, however, the infected larvae are torn open and the red spore masses are freely exposed to the air and light, the germination may involve practically all the spores. The germ tubes from adjacent spores may cohere in such a way that *Isaria*-like fascicles of conidiophores are produced. It appears, therefore, that although the germ tubes are capable of breaking out through the cuticula of the insect, the process of germination is facilitated considerably when the larva becomes disintegrated in the soil.

When the conidia are placed in contact with healthy insects, either externally or internally, infection may be readily induced. In all probability the conidia send out germ tubes that penetrate into the body cavity of the insect where they produce bodies that give rise to the yeastlike blastocysts. Thus, in summarizing what is known of the life cycle of *Sorosporella uvella*, it might be briefly said that the thick-walled resting spores germinate, producing conidiophores upon which are borne thin-walled conidia; the conidia in turn probably send out germ tubes, which, after invading the host insect, give rise to the vegetative cells known as "blastocysts," which multiply rapidly, ultimately becoming resting spores.

The resting spores do not necessarily require a long period of rest before germination, as is the case with certain other fungi. Germination may take place at once if suitable conditions are provided. The spores may be viable after being held in a dried state for 14 months, and they are able to withstand the low temperatures that prevail during the winter months in northeastern United States. Thus the resting spores are able to tide the fungus over periods of unfavorable conditions. The thin-walled conidia, on the other hand, apparently have the function of spreading the organism rapidly while favorable conditions persist.

S. uvella can be cultivated on a variety of nonliving media. Although its normal method of vegetative development within infected insects is by means of the yeastlike budding cells, on media a semifilamentous growth is obtained. As in nature, no perfect or ascigerous stage has so far been observed in any of the artificial cultures.

Distribution in Nature. Since its original discovery, *S. uvella* has been reported as a parasite of a number of lepidopterous and coleopterous insects in North America and in Europe. It has been reported from cutworms in Canada, and in the United States at least 10 insect species are known to be susceptible. In the United States it was originally collected in the field on the striped cutworm, *Euxoa tessellata* (Harr.). It has

also been found killing the corn earworm, *Heliothis armigera* Hb., in Virginia. Barber and Dicke (1937) report that high-humus-content soil appears to be very favorable to the development of the fungus and that, during a 5-year experimentation period, no moths of the corn earworm emerged from hibernation in this soil. In Florida considerable reductions in populations of mole crickets (*Scapteriscus*) have been reported through the agency *S. uvella* and the green-muscardine fungus, *Metarrhizium anisopliae* (Metch.). It is probably more widespread than is generally recognized; the relative inconspicuousness of the fungus probably accounts for its being overlooked in many cases.

In Moravia the poppy-root weevil, *Stenocarus fuliginosus* Marsh, is occasionally found parasitized by the fungus, as is the sugar-beet curculio, *Cleonus punctiventris* Germ. Some authorities (*e.g.*, Danysz and Wize, 1903) maintain that *S. uvella* is a more effective enemy of *Cleonus* than is *M. anisopliae.* Along with the green-muscardine fungus, *S. uvella* kills a considerable number of the larvae of the black beet weevil, *Psalidium maxillosum* Fabr., in the Russian province of Krasnodar. Most *Sorosporella* infections, however, are found in lepidopterous insects, the noctuids, *e.g.*, *Euxoa segetum* (Schiff.), being particularly susceptible. An accurate estimate as to the probable amount of natural control effected by this fungus has not been made. Since it has been shown that infection of larvae may result by various methods of inoculation (direct contact, feeding, and spray methods), it can be assumed that transmission in nature is probably readily maintained as long as all environmental conditions continue to be satisfactory.

Mycoses of Bees

A number of species of Fungi Imperfecti are known to be capable of parasitizing bees, particularly the honeybee, *Apis mellifera* Linn. That this beneficial insect suffered from mycoses was not generally realized until after the bacterial diseases of brood were recognized. The first comprehensive studies on the fungous diseases of bees were accomplished in Europe (see Toumanoff, 1930). Very little was done in this connection in North America until Burnside (1930) undertook a study of the fungi associated with the honeybee and their pathogenicity for this insect. Although there have been subsequent investigations and reports on this subject, our information is still meager, and much remains to be learned of the epizootiology of these diseases. One reason for this lack of attention is probably the fact that fungous diseases of bees appear to be less serious and less destructive than the common bacterial diseases of these insects. Furthermore the mycoses rarely become epizootic in intensity, although such outbreaks may occur among wild bees.

Fungi Concerned. In Europe, two species of fungi, *Aspergillus flavus*

Link and *Pericystis apis* Maassen, are recognized as the etiological agents of diseases of brood and adult bees. Since *A. flavus* attacks worker brood and adult bees, it is generally considered of greater economic importance than *P. apis*, which attacks only drone brood. When affecting brood the *A. flavus* infection is commonly known as "stone brood" (*Steinbrut*), and the *P. apis* infection as "chalk brood" (*Kalkbrut*). Other fungi that have been reported from Europe as capable of infecting and killing bees and their brood include *Trichoderma lignorum* Tode, *Mucor mucedo* Linn., and the yeast *Saccharomyces apiculatus* Hansen. Although not ordinarily pathogens of live bees, *Penicillium glaucum* Link and *Pericystis alvei* Betts are two of the most common fungi in European hives.

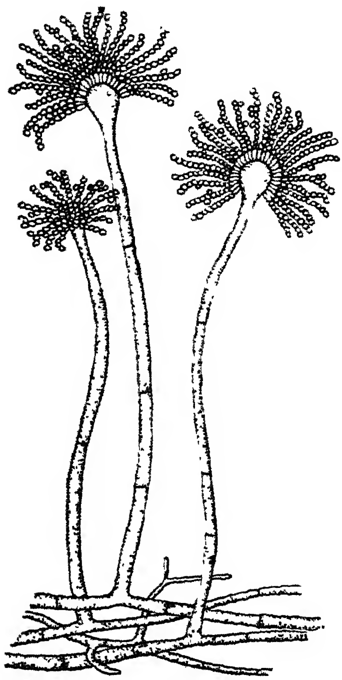


Fig. 129. *Aspergillus flavus* Thom & Church, which, under certain conditions, may cause a serious mycosis of brood and adult honeybees. (*Redrawn from Masera, 1936; after Pollacci and Nannizzi.*)

In the United States, *Aspergillus flavus* attacks brood of the honeybee more frequently than other fungi. It has also been recorded on five or six other species of insects in this country. *Pericystis apis* is not known to occur in North America. In addition to *A. flavus* other forms of *Aspergillus* known to attack bees include *A. fumigatus* Fres., *A. nidulans* (Eid.), *A. niger* Tieg., *A. glaucus* Link, and *A. ochraceus* Wilh. Burnside (1935) found *Mucor hiemalis* Weh. pathogenic for young bees when the latter are exposed to a temperature of about 20°C. By inoculation a 100 per cent mortality may be obtained with this fungus, of which the chlamydospores from infected bees appeared to be the most virulent form. A number of yeasts (*Saccharomyces*) are known to be pathogenic to the honeybee when introduced into its body cavity.

Nature of the Mycoses. During his experiments on the pathogenicity of various fungi associated with bees, Burnside (1930) observed several interesting aspects relating to the nature of the mycoses concerned. We shall repeat here the statement of some of his findings.

In the first place, Burnside observed that the greater number of fungous species that cause diseases of adult bees also attack the brood. He found

this to be the case with most of the species or strains of *Aspergillus* used in his investigations. When inoculated experimentally with a pathogenic *Aspergillus,* brood is attacked and killed more quickly than are adult bees, though the loss of brood resulting from *Aspergillus* mycosis is much less than that of adult bees. In general, mycoses of bees reach their greatest significance with adult workers. Throughout the active season, an appreciable number of these are killed by pathogenic fungi, principally by the yellow-green spored aspergilli. All races of bees common in the United States are susceptible.

The frequency with which bees are attacked by fungi in nature, according to Burnside, appears to depend chiefly upon the virulence of the pathogenic species and upon their dispersion. Conditions that favor abundant growth of pathogenic fungi in nature are conducive to the spread of fungous diseases. The fact that brood is rarely attacked can probably be explained by the small probability that the larval food will contain a sufficient number of viable spores to cause infection. The pathogenicity of a fungus apparently is determined by the ability of its spores and mycelium to resist the action of the intestinal fluids within the digestive tract of bees. Most writers on the subject seem to believe that infection is initiated by ingested spores, the infection progressing from the alimentary tract to the body cavity and internal tissues.

The appearance of brood dead of a fungous disease is fairly characteristic and easy to identify. The larva becomes noticeably harder soon after it dies, and the glistening white color changes to a dull creamy white. Later the dead insect becomes shrunken and wrinkled. The anterior end dries most rapidly, often curving upward at first, later tending to straighten out again. The fungus grows through the body wall, at first just back of the head in the form of a white ring or collar; a day or two later it covers the entire insect. As the fungus continues its development, spores are formed on the external mycelium giving it a green, yellow, black, or other color depending on the particular species of fungus concerned. As the spores and insect carcass become old and dry, the color usually fades.

When adult bees are infected with a fungus they become restless and weak. The weakness increases until death occurs. Some of the infected bees may die in the hive, but usually they fly or crawl away to considerable distances before dying. An increased firmness of the insect's body can sometimes be noticed at the time of death. It is usually more noticeable a few hours later. Under suitable conditions the external production of spores takes place, giving the insect a characteristic mealy appearance. Since nonpathogenic or saprophytic fungi may develop on the cadaver of a bee dead from another cause, care should be exercised in ascribing

the death of the insect to the fungus present, unless the examination is made directly after the death of the bee.

The exact cause of death has not been determined in the case of most of the mycoses, although the destruction to the host's tissue caused simply by the growth and development of the fungus is probably sufficient in most instances. There is evidence, in certain instances at least, that in addition to this physical action there is a chemical or toxic action exerted by the fungus. Undoubtedly a certain amount of digestion of the insect's tissues caused by the activity of fungous enzymes takes place. *Aspergillus flavus* has been shown to produce a transient toxic substance that is capable of causing a fatal poisoning in bees (Toumanoff, 1928; Burnside, 1930). In fact, Toumanoff (1931) believes that the pathogenic action of this fungus is due almost entirely to its toxic action.

Economic Importance. The importance of mycoses among bees is difficult to estimate accurately. Larvae may be carried out of the hive soon after they become infected, and adult bees usually die away from the hive. The mortality also varies somewhat according to the species of fungus involved. Some species attack adult bees in nature with considerable frequency; others attack the insects so rarely that under normal conditions their importance is negligible. Within the hive, the death rate may vary according to the condition and inherent strength of the colony. If wintering conditions are poor, allowing moisture to collect in the hive, and if the colony is weak, mycoses are likely to be a more serious threat than otherwise.

In general, it may be said that only slight losses of brood are caused by fungous diseases, and these are of negligible economic significance. Losses of adult bees from fungous infection are also of little economic importance, except that, when pathogenic fungi grow within the hive on combs, frames, dead bees, and the like, late in the winter or early in spring, significant losses are likely to occur. Such losses, however, can be largely remedied by exercising a few simple precautions.

Great care should be taken to ensure the proper ventilation of the hive and to provide for the escape from the hive of metabolic water vapor. Bees dead of fungi or covered with spores should be cleaned from the hive whenever this can be done without exposing the colony to severe weather conditions. All combs and equipment covered with fungi or "mold" should be washed or dipped for a few minutes in 20 per cent formalin, or other effective fungicide.

Other Fungi-Imperfecti Infections

The entomogenous Fungi Imperfecti are so numerous that it has been necessary to limit detailed discussion to a few of the better known ex-

amples. Furthermore it is impractical even to mention examples of all the remaining genera of imperfect fungi known to include entomogenous species.

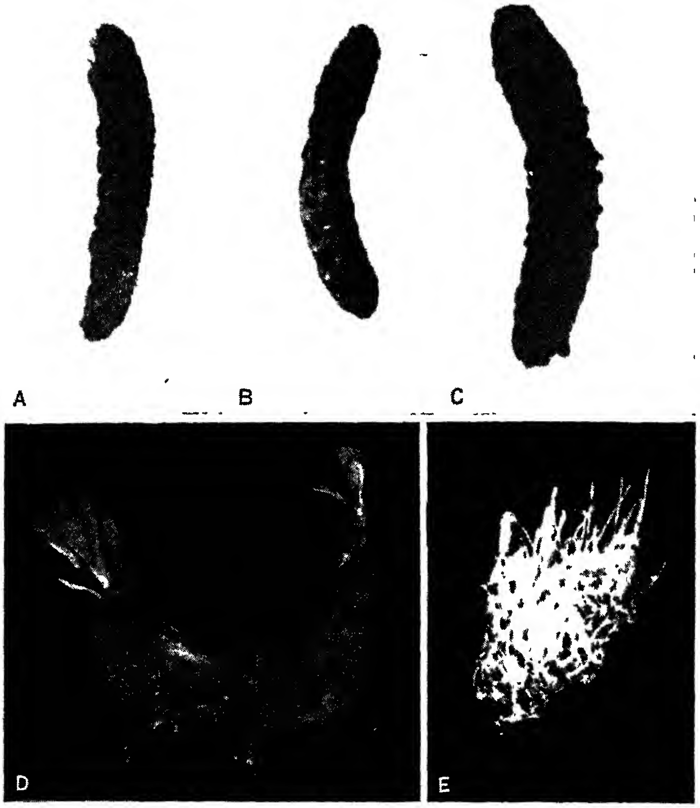


Fig. 130. *Spicaria* infections of cutworms. *A.* Larva of cutworm, *Feltia ducens* Wlk., infected with *Spicaria rileyi* (Farl.), showing only a trace of external mycelium. *B.* Larva showing further trace of external mycelium. *C.* Larva covered with mature spores of the fungus. *D.* Larva of bronzed cutworm, *Nephelodes emmendonia* (Cram.), infected with *Spicaria farinosa* (Fron.), showing rootlike subterranean outgrowths and spore-bearing hyphal fascicles. *E. Spicaria* sp. developing on a cutworm pupa. (*From Crumb, 1929; courtesy of U.S. Deparment of Agriculture.*)

Representatives of some of these miscellaneous genera include *Vermicularia cicadina* Ell. & Kell. on cicadas (some authors regard *Vermicularia* and *Colletotrichum* to be of the same generic type), *Monilia penicillioides* Del. on scarabaeids, *Trichoderma viride* Pers. on bees and culicids, *Cylindrodendrum suffultum* Petch on tipulid pupae, *Acrostalagmus aphidum* Oud.

and *Cladosporium aphidis* Thuem. on aphids, *Acremoniella verrucosa* Togn. on the clover leaf weevil, *Stemphylium botryosum* Wallr. on coccids, and *Synnematium jonesii* Speare on various Hemiptera.

Approximately six species of *Penicillium* have been reported as being parasitic on North American insects. About the same number of species of *Sporotrichum* have been similarly reported. The student should remember that the literature contains mention of a number of fungi, described or cited as species of *Sporotrichum*, most of which are in reality

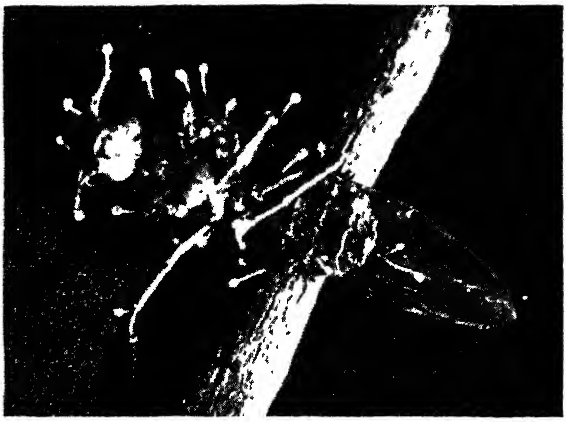


Fig. 131. A winged ant (*Camponotus*) infected with *Stilbum burmense* Mains (Moniliales: Fungi Imperfecti). The specimen was collected in Burma by W. L. Jellison. (*Photograph by K. M. Hughes.*)

synonymous with species of other genera such as *Beauveria* and *Acremonium* (*e.g.*, *B. globulifera* (Speg.) and *A. tenuipes* Petch). The genus *Rhinotrichum* contains at least one American species of entomogenous fungus (*R. depauperatum* Charles) parasitic on the avocado red mite. Members of the genus *Hormodendrum* are rarely pathogenic for healthy insects but are found occasionally on weakened or dead insects. In the United States, *Macrosporium* has been reported on certain Coleoptera, Lepidoptera, and Hemiptera. *Syngliocladium* has been found on wireworms.

The genus *Spicaria* contains numerous entomogenous species, one of which, *S. farinosa* (Fron) Vuill., was mentioned earlier in this chapter. It was with this species, as the type, that Petch (1934) proposed that the name *Isaria* be discarded in favor of *Spicaria*, *Isaria farinosa* (Dicks.) Fron becoming *Spicaria farinosa* (Fron) Vuill. The generic name *Isaria* had been used generally by mycologists in connection with the conidial stages of *Cordyceps*. About a dozen species of *Spicaria* have been reported as parasites of insects in North America. Among these, in addition to *S. farinosa*, are *S. heliothis* Charles on the corn earworm, *S. canadensis*

Vuill. on larvae of the satin moth, and *S. rileyi* (Farl.) on a number of Lepidoptera and Coleoptera. The genus *Nomuraea* is generally considered as a synonym of *Spicaria*. Another genus whose species are imperfect stages of *Cordyceps* is *Hymenostilbe*, of which at least six species have been found on North American insects.

BASIDIOMYCETE INFECTIONS

The most remarkable association between insects and Basidiomycetes concerns those fungi belonging to the genus *Septobasidium*. Beneath the stromata of these fungi live certain scale insects, some of which are parasitized by the fungi. Because of the latter fact some authors consider the general relationship between the fungi and the insects to be one of parasitism. On the other hand, one of the leading students of the genus *Septobasidium*, Couch (1931), considers the relationship to be one of mutual symbiotism, in which the parasitization of some of the insects is for the good of the colony. In accordance with this view of Couch's, we have discussed this group of fungi in Chap. 4, along with other extracellular microbiota of insects. Since the parasitism that does take place is also discussed there, we shall omit further reference to the genus in the present chapter.

The number of known basidiomycete fungi truly parasitic on insects is comparatively small. Certain species in the genera *Helicobasidium*, *Corticium*, *Thelophora*, *Hymenochaete*, and *Daedalea* are now considered synonymous with members of the genus *Septobasidium*. Intermediate between the rusts and the genus *Septobasidium* has been erected the genus *Uredinella* of which the type species, *U. coccidiophaga* Couch, has been found on specimens of *Aspidiotus*. The imperfect genus *Hirsutella* was at one time thought to be a basidiomycete (Agaricales), but most of the species now contained in it are considered to be the imperfect stages of certain Ascomycetes, *e.g.*, certain *Cordyceps*.

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CHAPTER 11

VIRUS INFECTIONS

Knowledge of viruses in general began with the work of Iwanowski and that of Beijerinck, who toward the close of the last century observed that the mosaic disease of tobacco plants could be reproduced in healthy plants with bacteria-free filtrates of the juice from affected plants. About this same time, Löffler and Frosch demonstrated the filterability of the agent of foot-and-mouth disease of cattle. Soon thereafter, diseases caused by filterable agents were recognized in man and other vertebrates, in invertebrates, and in numerous plants.

Since no particles or organized elements could be seen in the infectious filtrate, Beijerinck called it a *contagium vivum fluidum.* The amazing thing about this material, besides the fact that nothing could be seen in it with an ordinary microscope and that nothing grew when it was planted on ordinary culture media, was that it would pass through the pores of thick porcelain filters that retained bacteria and yet remain infectious and cause reproducible disease. Because of this property, the agent concerned was called a "filterable" virus, the word "virus" being used in the general sense meaning an infectious agent. Later it became evident that filterability could not be used as a hard and fast criterion since not only are some of the visible bacteria small enough to pass through filters but several extremely small agents are not filterable. Accordingly, the term "filterable" is now generally dropped and the word "virus," by itself, is considered synonymous with "filterable virus."

As a group, most viruses have properties and characteristics that are fairly distinctive. In size, they range from about 10 to almost 300 millimicrons in diameter—a few have lengths in the neighborhood of 400 millimicrons. They react to chemical and physical agents in a manner similar to that of bacteria. The viruses of plants and of higher animals have a strong tendency to vary or mutate; *i.e.*, strains frequently arise that differ to some extent from the parent strain. Another feature that sets some viruses apart from other infectious agents is their apparent crystalline structure. A few viruses have been crystallized and obtained in a relatively pure chemical state that reveals them to be protein in nature. As yet, none of the viruses that cause diseases in insects has been crystallized. Viruses are also distinguished by the fact that they are obligate parasites.

They cannot be grown in the absence of living cells, and their life processes are apparently dependent upon the metabolism of the host cell. That they are morphologically distinct entities has been revealed by the electron microscope, which shows them to possess shapes analogous to those of bacteria.

The viruses that cause diseases of insects are in general similar to most other viruses in their basic properties. Many of them do, however, possess certain characteristics that distinguish them from those causing diseases of higher animals and plants. One of these characteristics is the production of peculiar crystallike bodies, called "polyhedra" (Gr. *polys* = many + *hedra* = seat, side), in the cells of tissues affected by the virus. However, not all virus diseases of insects are characterized by the formation of polyhedra, although in the majority of cases so far known these cellular inclusions are in evidence. Instead of producing polyhedra, some viruses cause the formation of refringent inclusions of varied sizes and shapes. Others manifest their presence by small granular inclusions, or "virus capsules," which enclose the virus and which fill the cytoplasm of the infected cells and which accompany a generalized breakdown of the cell constituents, including the nucleus. Still other insect viruses are characterized by the complete absence of inclusions of any kind. Thus the virus infections of insects may be divided into at least four large groups:

1. Those characterized by the presence of polyhedral inclusions
2. Those characterized by the presence of refringent polymorphic inclusions
3. Those characterized by the presence of granular inclusions ("virus capsules")
4. Those not characterized by the presence of any kind of inclusion bodies

The first two of these groups, and sometimes group three, are referred to by some authors as "nuclear diseases" because the nucleus is an early seat of the infection and because some of the most significant pathological changes of these diseases take place in the nuclei of the infected cells.

In addition to these four groups, the literature contains occasional mention of certain ill-defined and poorly described disease conditions considered by their authors to be caused by "viruses." An example is the dropsy or "*Wassersucht*" described by Heidenreich (1939) in larvae of *Melolontha melolontha* (Linn.) (= *M. vulgaris* Fabr.) and *M. hippocastani* Fabr. Among other things this disease is characterized by a liquefaction of the adipose tissue and by the presence of certain minute coccuslike bodies in the infected tissues. Another example, also described by Heidenreich, is a so-called "virus disease" of nun-moth pupae (*Lyman-*

tria monacha Linn.) in which there occurs a peculiar alteration of the adipose tissue. Diseases such as these must await further and more detailed study before we can classify them intelligently.

Classification and Nomenclature of Insect Viruses. The four arbitrary groups just delineated may in a sense be considered as indicative of four taxonomic groups of at least generic rank. It is not yet possible, on the basis of information now available, to ascertain with certainty the true phylogenetic relationships among these groups. It may be desirable and practical, however, to establish some sort of nomenclature by which the separate viruses of each of the four groups could be designated and referred to in literature. First let us examine past efforts in this direction.

Nomenclatorial practice with respect to the supposed causative agents of insect-virus diseases was first invoked with the application by Bolle (1894) of the name *Microsporidium polyedricum* to the polyhedron characterizing the disease in silkworms, *Bombyx mori* (Linn.), known as jaundice (*grasserie*, *Gelbsucht*). Bolle (1898) believed the "*polyedrischen Körperchen*" represented spores of a protozoan parasite that multiplied in a manner similar to that of coccidia. Von Prowazek (1907) demonstrated that the infectious agent would pass through filters capable of retaining the polyhedra. Although later abandoning the idea, for a time at least he believed that the infectious agent was a protozoan, which he named *Chlamydozoon bombycis*, and that the polyhedra themselves represented reaction products or by-products of the infection. A similar concept was later defended by Prell (1918, 1926), who believed that the granules seen within the polyhedra were the nuclei of the causative agent also located inside the polyhedron. For this parasite, as he conceived it, Prell proposed the genus *Crystalloplasma* with *Crystalloplasma polyedricum* (Bolle) as the type species. In 1926 he added a second species, *Crystalloplasma monachae*, the causative agent of the polyhedrosis (*Wipfelkrankheit*) of the caterpillar of the nun moth, *Lymantria monacha* Linn.

In the meantime, another concept as to the nature of the agent of silkworm jaundice was developing, beginning with the work of Acqua (1918–1919), who showed that the cause of the disease was a filterable agent invisible with an ordinary light microscope. Some of the most convincing support for this idea came from the work of Paillot (1924*a*, 1926*c*, 1930*b*), who was particularly concerned about certain minute granules demonstrable with the aid of a dark-field microscope in the jaundice-diseased silkworms. Paillot considered these ultramicroscopic granules to be the true cause of the disease and gave them the name *Borrellina bombycis*, type species of the genus *Borrellina*, which honored the name of the French bacteriologist A. Borrel. At the same time Paillot (1926*c*) placed two other species, *Borrellina pieris* and *Borrellina brassicae*,

in the same genus. The latter two names were used to describe agents that caused inclusion diseases distinct from the polyhedroses as characterized by the silkworm jaundice. Later Paillot (1933) added the name *Borrellina flacheriae* to designate the virus he considered to be the primary agent of the silkworm disease known as "gattine." Paillot considered this genus as indicative of a distinct group of ultramicroscopic agents intermediate between the bacteria and the protozoa.

Then, in 1929, appeared one of the most perplexing and incongruous papers ever published in the field of insect pathology. We refer to a 315-page treatise on the "giallume diseases" by Del Guercio, who thought himself to be concerned with the polyhedral infections of numerous insect pests of Italy. He gave the name "entomococci" to the causative agents, which he believed to be organisms phylogenetically located "between fungi and algae on one side and true bacteria on the other," and represented by fruiting bodies of polyhedric form. He characterized them as being microscopic, thallus-shaped, arborescent vegetative growths having a ramified and involved structure growing out of stromata. Some of the forms described presumably have small coccuslike reproductive forms.

To say the least, it is extremely difficult to appraise Del Guercio's work. It is almost completely contrary to many of the well-established facts concerning polyhedral infections as we know them today or as they were generally known at that time. Del Guercio himself points this out while discounting most of the early concepts as to the nature and development of polyhedra. Most of the large number of drawings with which he illustrates his paper appear to represent either very unusual situations and forms, or else they are artifacts of one sort or another; at any rate it is difficult to imagine just what it was that Del Guercio saw in his preparations. We are inclined to suggest that the student delay serious consideration of Del Guercio's observations until their true significance, if any, can be ascertained.

The part of Del Guercio's (1929) work that concerns us here is the nomenclature he introduced to designate the various forms he described. Seventy-one species are represented, of which Del Guercio gave names to 66. These species were separated into 12 genera, with the type genus being *Entomococcus*. The type species of the genus was *Entomococcus bombycinus* Del Guercio and was described from the silkworm host, *Bombyx mori* (Linn.). It seems hardly necessary to give all the names proposed by Del Guercio any further serious consideration, since it is quite obvious that whatever were the forms that he named they certainly were not viruses and probably had no phylogenetic relation whatever

to the agents causing polyhedroses. Therefore, as far as the systematics of insect viruses are concerned, Del Guercio's efforts in this direction may be removed from practical consideration.

In the sixth edition of "Bergey's Manual of Determinative Bacteriology," Holmes (1948) has presented a classification covering most of the known groups of animal and plant viruses and, following an earlier idea (Holmes, 1939), has instituted the use of binomials to distinguish each "species" of these agents. According to Holmes's presentation, the insect viruses, *i.e.*, the viruses causing diseases of insects, comprise two genera (*Borrelina*[1] and *Morator*) in the family Borrelinaceae, suborder Zoophagineae, order Virales. The genus *Borrelina* is characterized as being comprised of "[viruses] inducing polyhedral, wilt, and other diseases; hosts, Lepidoptera, so far as known." The genus *Morator* is described as follows: "Only one species at present, inducing the disease known as sacbrood of the honey bee."

The information and knowledge at hand concerning the insect viruses would probably have permitted Holmes to be less conservative than his presentation indicates. His statement that members of the genus *Borrelina* are "Known only as attacking lepidopterous insects" is true for the species he recognizes but unnecessarily excludes the rather well-known polyhedroses of diprionids in the order Hymenoptera, as well as the reported but little known polyhedroses of certain Diptera. Similarly, his statement that members of the genus *Morator* are "Known only as attacking the honey bee" might conceivably be broadened to include the virus (*Borrelina flacherie* Paillot) believed to be responsible for initiating gattine and true flacherie of the silkworm, a lepidopterous insect. Furthermore, the inclusion of *B. brassicae* Paillot and *B. pieris* Paillot in the genus *Borrelina*, as done by both Paillot and Holmes, might be questioned on the basis both of differences in the appearances of the inclusion bodies that characterize them and of the distinct natures of the diseases they cause.

In order to help clarify the situation with regard to the systematics of insect viruses, the writer proposes that until more definite information is available, these agents be considered as consisting of four principal groups according to the type of disease they cause and the type of inclusion bodies formed when the latter are present. These four natural groups have already been indicated in the preceding section. For practical and taxonomic reasons it would appear that each of these groups warrants at least generic status. In any case it would appear that a closer relation-

[1] Holmes has interpreted Paillot's original spelling of this genus with two l's as an error. Accordingly, he changed the spelling from *Borrellina* to *Borrelina*.

ship exists between one virus and the other members of its group or genus than between that virus and any of the other groups or genera. Accordingly, the following orientation, to be considered as tentative, is proposed:[1]

Family BORRELINACEAE Holmes, 1948

Viruses causing infections in arthropods, particularly insects.

Key to Genera

I. Viruses causing insect diseases characterized by the presence of polyhedral inclusions in the infected cells of the host.
   Genus I. **Borrelina** Paillot, 1926*d* (see pages 423 to 495)
   (Type species: *Borrelina bombycis* Paillot, 1926*d*)

II. Viruses causing insect diseases characterized by the presence of refringent polymorphic inclusions of very irregular shape and size in the infected cells of the host.
   Genus II. **Paillotella** *gen. nov.* (see pages 497 to 500)
   (Type species: *Paillotella pieris* (Paillot) comb. nov. [= *Borrelina pieris* Paillot, 1926*d*])

III. Viruses causing insect diseases characterized by the presence, in large numbers, of very small but microscopically discernible granular inclusions in the infected cells, particularly visible in the cytoplasm, of the host. These granules consist of proteinaceous material within which the virus particle is located.
   Genus III. **Bergoldia** *gen. nov.* (see pages 500 to 514)
   (Type species: *Bergoldia calypta* spec. nov.)

IV. Viruses causing insect diseases in which no visible pathological inclusion body of any kind is produced.
   Genus IV. **Morator** Holmes, 1948 (see pages 516 to 536)
   (Type species: *Morator aetatulae* Holmes, 1948)

Of the two new genera proposed here, the name *Paillotella* is after André Paillot, who discovered the only known species of the genus, and the name *Bergoldia* honors Gernot Bergold who first conclusively proved the virus nature of the agents included in this genus when he demonstrated the virus particles of the granulosis of *Cacoecia murinana* Hüb.

[1] The author is aware of the inappropriateness, in many instances, of erecting new names for taxonomic categories in a book of this kind. In this particular case, however, it is felt that the proposal being made requires the background of information that only a detailed treatment of insect viruses, such as given in the present chapter, can supply. To make this proposal at the present time in a separate short taxonomic paper would be likely to leave the subject inadequately treated and not fully clarified. In the present chapter, full and detailed descriptions of the generic characteristics are given in the respective sections.

It is entirely possible that, as our knowledge of insect viruses increases, the concept just presented will have to be modified, but for the present it at least provides a workable starting point. With regard to the naming of the species in these genera, it seems best for the time being to proceed cautiously and, when possible, to include in the specific descriptions the appearance of the virus as seen with the electron microscope. This would avoid such situations as that in the case of *Borrelina bombycis*, for example, which has been described as a coccuslike body or granule when, in reality, the virus is an elongated rod-shaped body approximately 40 by 288 millimicrons in size.

The marked variability or mutability seen in so many viruses pathogenic for higher animals and plants has not been well studied as far as the viruses pathogenic for insects are concerned, although such has been claimed in one or two cases (*e.g.*, Paillot, 1941*b*). Undoubtedly such variations do exist, but since their detection usually depends on differences in symptoms produced in the host—and the symptoms of virus diseases in insects are usually not well defined—natural variations of insect viruses have, for the most part, been overlooked.

VIRUS DISEASES CHARACTERIZED BY THE PRESENCE OF POLYHEDRAL INCLUSIONS

(Polyhedroses or Polyhedries)

Although approximately 100 species of insects are known to be susceptible to viruses that cause polyhedra to appear in the cells of the infected tissues, this is probably only a portion of the number that do exist. Undoubtedly a large number of viruses and a large number of susceptible hosts remain to be discovered and recorded, since the subject is one that has had only a smattering of investigation and observation—some very excellent and some very superficial. Furthermore, much remains to be learned about even those virus diseases which are well known and of which we now have considerable information, and in these cases John Heywood's proverb that "Much water goeth by the mill that the miller knoweth not of" is applicable.

Diagnosis of a polyhedral virus disease is made relatively easy by the fact that one has but to make a microscopic preparation of a diseased tissue and, if polyhedral bodies are present, it is a certainty that an infecting virus is also present. The opposite of this is also apparently true: whenever the virus invades the tissues of a susceptible insect, polyhedra may be seen to appear in the affected cells. Within a short time after the infected insect dies its disintegrating tissues are literally filled with polyhedra, which may be seen suspended in large numbers in the body fluids. It is no wonder that the attention of early investigators was

drawn to these bodies, which they at first supposed represented the true cause of the disease and which were, in fact, thought to be protozoan in nature. As soon as it was finally determined that the polyhedral bodies were not, in themselves, living organisms, the question arose as to what they really were and what part they had in the obviously diseased condition of the insects in which they were found.

Even today it is difficult to give a clear definition as to the exact nature and genesis of polyhedra. Among that which is known about them is this: they need not be present to initiate the disease; *i.e.*, the virus itself is infectious in the absence of polyhedra, and it may be separated from the polyhedra by filtration and by dissolving away the polyhedral protein with weak alkalies. The electron microscope has revealed with certainty the identity of the virus as distinct from the polyhedra; the polyhedra *in toto* have never with certainty been entirely freed of the virus, initial claims in this regard having been subsequently retracted. The explanations for this are varied. Some believe that the polyhedron is actually composed of virus particles; others think that virus particles are fortuitously incorporated within the crystalline structure of the polyhedron when it is formed, and still others assume that it is merely a case of very strong adsorption of the virus particles onto the polyhedron. In the light of recent observations, however, it is now believed that the virus particles, in considerable numbers, occur singly or in bundles within the polyhedral body itself. The polyhedra range in size from about 0.5 to 15 microns, while the virus particles have known sizes in the neighborhood of 200 to 400 by 40 to 80 millimicrons.

The great majority of the hosts of known polyhedral viruses are species of Lepidoptera. A significant number of Hymenoptera and a few Diptera are also known to be susceptible to this type of infectious agent. What the inherent physiological properties are that determine the marked resistance of such groups as Orthoptera, Coleoptera, and Hemiptera is not known. Furthermore, in those groups, members of which are subject to attack by these viruses, usually it is only the immature stages, particularly the larvae, that are highly susceptible. There have been some reports of polyhedra being present in the tissues of adult insects, but it is questionable whether these represent active or frank infections. It would be of great biological interest to know just what takes place in the tissues of insects to change their susceptibility to insusceptibility as they reach the adult stage. Details concerning the host range of polyhedral viruses will be brought out later in our discussion. Let us first review briefly some of the more general aspects of entomophilic viruses, beginning with the classic polyhedrosis of the silkworm. Since undoubtedly more is known about the polyhedrosis of the silkworm than about that of any

other insect, and since the information that has been gathered concerning this disease is, in general, applicable to most other polyhedroses, the student will do well to familiarize himself with the silkworm disease, even though he may be interested primarily in the polyhedroses of destructive insects. The reader searching the literature for the general principles relating to polyhedral diseases will find most of them stated in terms of the discoveries made during the course of investigations on silkworm jaundice. It is therefore of value to consider this disease from somewhat of a chronological and historical viewpoint.

Jaundice of the Silkworm

Polyhedrosis of the silkworm, *Bombyx mori* (Linn.), is known under a variety of names, depending upon the language of the country concerned. Thus it is called "jaundice" in America, "*grasserie*" in France, "*giallume*" in Italy, "*Gelbsucht*" in Germany, etc. The names indicate either the yellowish color of the diseased insect or its temporarily distended, swollen, or fatlike appearance. A caterpillar in the latter condition is *engraissé*, hence the French name "*grasserie*," which is as much in common use as is the name "jaundice."

Other symptoms characteristic of jaundice include the silkworm's loss of appetite and its marked inactivity. Just before death the integument becomes opaque and assumes a shiny, yellow, or brownish color. The larva is entirely flaccid and usually without offensive odor; the internal tissues are in a state of disintegration. The caterpillar is extremely difficult to remove without breaking the skin and liberating the liquefied contents. The time from infection until death averages from 6 to 8 days.

The disease has been known for a long time, but, at that, it has frequently been confused with other diseases of the silkworm, and its identity as a separate entity was definitely established only after the characteristic polyhedral bodies were recognized. The Italian poet Vida, as early as 1527, probably refers to this disease in his poem "De bombyce," when he mentions the afflictions of the silkworm. It is also mentioned in a book on butterflies written in 1679 by Maria Sibylla Merian. One of the earliest published descriptions of the disease itself is that of Nysten in 1808. For many years, however, jaundice was considered a disease of only minor importance. In fact, in some localities sericulturists frequently thought that the presence of a few jaundiced silkworms in their nursery was an indication that they would obtain an excellent yield of silk-spinning caterpillars. Eventually it became apparent that the disease was of a very destructive nature and that at times it could produce losses more serious than those caused by the other well-known diseases of this insect.

The Cause of Jaundice in the Silkworm. As we have already indicated, until the last few years of the nineteenth century jaundice of silkworms was confused with other diseases of this insect—flacherie, gattine, muscardine, and pebrine. By 1890, however, many sericulturists were recognizing it as a more or less distinct entity, although there was no general agreement as to its etiology. Among the various amicrobial factors ascribed to its cause were such things as poor nourishment, careless wintering of the eggs, uneven temperatures, damp air, poor ventilation, and excessive moisture. Today we know that some of these conditions, though not the specific cause of the disease, do influence the course of the infection and constitute important predisposing causes.

Microbial agents were also suspected of being responsible for the disease; and, as was the case in other virus diseases, several species of bacteria were isolated and designated as the causative agent. Krassilstschik, in 1896, ascribed the cause of the jaundice to a bacterium, *Micrococcus lardarius*, which he found in the hemocoele and alimentary tract of diseased silkworms. This organism, like others (*e.g.*, *Micrococcus bombycis* Cohn), proved to be a mere secondary invader without any specific relation to the true disease agent.

Now, in the meantime, several early investigators had observed peculiar crystallike corpuscles or bodies in the tissues and body fluids of diseased silkworms. Cornalia (1856) and Maestri (1856) were apparently among the first to make such observations and to associate these bodies with the disease. Cornalia described some of the pathological manifestations of the disease and reported that the polyhedral bodies in the blood corpuscles originated from some kind of alteration of the blood. Maestri also observed the polyhedral bodies in the blood cells as well as in other tissues and called attention to their location in the nuclei of the cells. As far as the characteristic dissolution of the tissues was concerned, Maestri believed that the action of heat on the respiratory system of the silkworm brought about an alteration and a complete melting of the adipose tissue. Some years later Haberlandt (1871) referred to the polyhedra as crystals, and Verson (1872) and Panebianco (1895) studied them from a crystallographic standpoint and likened them to rhombododecahedral crystals. Bolle (1894), at first, also considered them as crystals; then he decided that these "*polyedrischen Körperchen*" represented the sporulated form of a protozoan parasite to which he gave the name *Microsporidium polyedricum*. He (1898) believed it to be a sporozoan that multiplied in a manner similar to that of coccidia, and his drawings depict a coccidianlike oöcyst filled with polyhedra, which he apparently believed were sporocysts. Bolle's views were supported by Marzocchi (1908). Sasaki (1910), however, discounted Bolle's theory and believed that the polyhedra could arise

from a variety of causes and that they represented the degeneration or atrophy of the nuclear contents of the infected cell.

In 1907 von Prowazek made a significant advancement when he found that material from diseased silkworms was still infectious after the polyhedra had been removed by filtration through several layers of filter paper. This worker at first conceived the idea that the disease was caused by a parasitic protozoan, which he designated by the name *Chlamydozoon bombycis*, and the polyhedral bodies represented reaction products or by-products of the infection. This concept was subsequently modified by Prell (1918, 1926), who believed that the granules observed inside the polyhedra were nuclei of the causative agent, which he called *Crystalloplasma polyedricum* (Bolle). Later, in 1912, von Prowazek did not emphasize his chlamydozoan theory and apparently was in a mood to abandon it. Whatever the cause, however, he believed that the polyhedra were simply by-products of the disease. Von Prowazek's earlier views were supported by Wolff (1910), who had studied a polyhedrosis of *Bupalus piniarius* Linn., and to the cause of the supposed "chlamydozoonosis" of this insect he gave the name *Chlamydozoon prowazeki*. Wolff also believed that bacteria, especially certain streptococci, had a synergistic effect on the chlamydozoa and thus indirectly helped precipitate the disease.

Shortly before von Prowazek published his 1912 paper, an interesting surmise as to the cause of a similar disease in larvae of the nun moth was advanced by Escherich and Miyajima (1911), who stated the belief that the disease was caused by an unknown virus for which the polyhedron acted as a carrier. Shortly thereafter, Hayashi and Sako (1913) came to a like conclusion with regard to the silkworm disease. Convincing proof that the cause of jaundice in silkworms was a filterable virus, invisible with an ordinary microscope, came with the work of Acqua in 1918–1919. Support for the filterable-virus idea was forthcoming through the work of Paillot (1924*a*, 1926*b*, 1930*b*) and through that of other investigators working with similar diseases in other insects. To the agent of jaundice, which he believed was represented by tiny granules visible with a dark-field microscope, Paillot gave the name *Borrellina bombycis* (now spelled *Borrelina bombycis*) in honor of Professor Borrel of the Pasteur Institute.

Parenthetically, it should be mentioned that during the first third of the present century other ideas as to the cause of jaundice were being advanced in spite of the work of the investigators mentioned. In 1925, for example, Nello-Mori explained the origin of the virus by assuming that it was the filterable form of larger microorganisms such as yeasts. Pospelov and Noreiko (1929) concur with Nello-Mori and believe they have shown that the viruses causing insect diseases are ultramicroscopic involution forms of yeasts. These workers claim to have initiated polyhedroses by

feeding the yeast *Debaryomyces tyrocola* to larvae of various lepidoptera, including the silkworm. Pospelov (1929) further believes that the polyhedra are breakdown products of the nuclei of the rapidly dividing cells of sick caterpillars. The small granules which eventually become polyhedra and which may be seen in the nuclei of infected insects, Pospelov believes represent symbiotes that are always present in small numbers, even in healthy larvae. He also believes that under certain "circumstances and stimulation they increase and are destructive and become the polyhedra." It is somewhat difficult to follow Pospelov's theory, which has not been substantiated by the work of most investigators of these diseases and which must be considered as unproved. Certain other Russian workers (*e.g.*, Rischkow) apparently do not consider Pospelov's theory as tenable.

On the basis of all our present information and data we may conclude that jaundice of silkworms is caused by an ultramicroscopic virus, that it is a parasite principally of the nuclei of certain cells, and that it initiates the morbid process that ends in the elaboration of polyhedral bodies. Let us now consider the nature and characteristics of the virus and its relation to the polyhedra.

The Virus of Silkworm Jaundice. That jaundice may be initiated in silkworms in the absence of polyhedral bodies is known with certainty. As we have already indicated, this was shown as early as 1907 when von Prowazek reported that jaundice material is still infectious after the polyhedral bodies are removed by filtration through several layers of filter paper. This fact has since been confirmed by filtration experiments which showed that the infecting agent passes through Berkefeld V and N, and Chamberland L1 and L2 filter candles, all of which retain the polyhedra, although filters having a porosity as fine as the Chamberland L5 candle retain it. Furthermore it is possible to transmit the causative agent by blood from a diseased larva before the appearance of polyhedra in the tissues of the infected insect. We may first, therefore, logically consider the nature of this filterable agent before dealing with its relation to the polyhedra.

Beginning with von Prowazek's observations, the presence of minute granules or particles has been reported frequently in the blood and tissues of diseased caterpillars. Whether all investigators have been talking about the same granules is not clear. At any rate, the French worker Paillot was a leading proponent of the etiological nature of certain small granules, invisible by ordinary microscopy but visible with the aid of a dark-field microscope, which occur in the blood and tissues of diseased silkworms. These granules are always less than 0.1 micron in diameter and are animated by vigorous Brownian movement. According to Paillot (1924*a*,

1926*b*), they are visible first in the cytoplasm of the blood cells, where they may be seen in the interior of small liquid spheres that often rupture at the surface of the cell; they then penetrate into the nucleus, where they multiply abundantly, forming an easily visible, shining ring. The question arose: are these granules the virus of jaundice? Paillot believed them to be and, as we have already mentioned, named them *Borrellina bombycis* (now spelled *Borrelina bombycis*). Similar particles, however, may be seen in the hemolymph of healthy caterpillars. Paillot (1943) maintains that the latter particles differ in some respects from those observed in diseased insects, primarily by occurring in fewer numbers. Glaser and Cowdry (1928), on the other hand, studied the granules visible in healthy and diseased caterpillars from both a quantitative and a qualitative standpoint. These workers were unable to see any marked difference between the minute bodies in normal blood and those in diseased blood. They doubted that those granules in the blood of diseased insects represent the true virus, which they believed to be invisible with an ordinary light microscope or with a dark-field instrument. A year before this, Glaser (1927) had pointed out that when normal cells of the silkworm are allowed to degenerate, they also show minute violently vibrating granules within the cytoplasm and nuclei. Paillot, however, insisted on maintaining his original concept of the virus, and certain other workers (*e.g.*, Letje, 1939, 1940*a*,*b*) supported him in this. The entire question was again highlighted by new data made available by more modern equipment and techniques.

In 1939, Gratia and Paillot reported that they were able, by fractional ultracentrifugation, to isolate the minute granules found in the hemolymph and tissue fluids of jaundice-diseased silkworms. They found these bodies, as well as the polyhedra, to be serologically distinct from the tissues that harbor them. Paillot and Gratia (1939), in a second paper, state that they were able to isolate minute particles from normal tissues, but not from normal blood, and that they were different in several respects from those found in diseased insects. They further explain that both normal and diseased blood have certain large coarse granules but that only the blood of the diseased specimens has the very minute granules characteristic of the disease. These have a size of about 100 millimicrons.

When the blood from an infected silkworm is centrifuged in an ordinary centrifuge (5,000 r.p.m. for 10 minutes), four layers may be distinguished. The bottom layer is a whitish sediment made up of polyhedra. Next to the bottom is a yellowish layer of cellular debris. Then there is a layer of serum, which is distinctly opalescent. On the surface of all this is a pellicle of oil, which may easily be lifted off with a forceps moistened in water. After separating the supernatant serum from the sediment by

another centrifugation, Paillot and Gratia subjected the clear serum to ultracentrifugation in a Huguenard centrifuge at 60,000 r.p.m. for 10 minutes. Similar treatment was given the blood from healthy silkworms. Both serums leave a sediment after ultracentrifugation, but this deposit is of a very different nature in the two cases. That of the normal serum is essentially a transparent mucous but compact layer that breaks up into small flaky masses when triturated. Under a dark-field microscope it appears to have a fibril consistency if the serum is fresh and a coarsely granular consistency if the serum is some days old. On the other hand, the sediment from the infected serum is opaque, yellowish, and of a powdery consistency. With the dark field it may be seen to be made up entirely of small characteristic granules.

In experiments to demonstrate the virulence of these granules, Paillot and Gratia obtained results that indicated the following: After ultracentrifugation of the serum from infected silkworms, the supernatant liquid had lost its virulence. The sediment, resuspended in physiological saline and returned to its original volume, is almost as virulent as the serum of the same blood. In other words, according to Paillot and Gratia, virulence appears to accompany these minute granules. While they were thus apparently able to separate off a virulent protein from the blood of diseased caterpillars, Paillot and Gratia, like Glaser and Wyckoff (1937), also obtained a homogeneous heavy, but noninfectious, protein from the tissue fluids of healthy silkworms.

In 1943, Glaser and Stanley reported on their attempts to concentrate and purify the virus of jaundice by ultracentrifugation. After twice centrifuging the polyhedra-free serum for 120 minutes at 27,000 r.p.m. in an ultracentrifuge with an 8-inch rotor, these workers obtained what they considered to be purified virus material. This was examined in an analytical ultracentrifuge by Lauffer (1943), who reported the presence of a single component having a sedimentation constant $s_{20}^{w} = 17$ S (Svedberg unit, a rate equal to 10^{-13} centimeter per second in a unit centrifugal field). The purified material was stable at 4°C. and gave the usual reactions to tests for proteins; it contained phosphorus and exhibited no double refraction of flow. Chemical analysis showed it to have a composition similar to that which has been obtained for other virus nucleoproteins. A portion of the purified material was examined by an RCA type B electron microscope. With this instrument, a micrograph of the purified material showed the presence of spherical particles about 10 millimicrons in diameter. Thus Glaser and Stanley conclude that the chemical and ultracentrifuge data indicate that the purified preparations from infected silkworms are nucleoprotein in nature and consist of spherical particles having a diameter of about 10 millimicrons and a molecular weight of

about 300,000. (As will be explained later, however, this material is now known to be not identical with the virus of silkworm jaundice.)

Up to this point, except for a few minor details, the findings of Glaser and Stanley and those of Paillot and Gratia are fairly compatible. In other respects, however, significantly different results were obtained by these two groups of workers. Instead of finding, as did Paillot and Gratia, that the purified material from normal caterpillars was distinctly different from that obtained from diseased insects, Lauffer found the sedimentation constant of Glaser and Stanley's purified material from normal blood to be essentially the same (17 S) as that of the material obtained from the blood of jaundiced silkworms. The gross chemical and physical properties of the material from normal and diseased blood were also the same. [The purified material, obtained in this instance from normal silkworms, apparently is not the same as that studied earlier by Glaser and Wyckoff (1937). The latter material is not stable under refrigeration, as is the material isolated by Glaser and Stanley.]

Since Gratia and Paillot (1939) [and later Bergold and Friedrich-Freksa (1947)] found the purified material from the blood of jaundiced caterpillars to be serologically different from the purified material from the blood of healthy caterpillars, Glaser and Stanley made a similar comparison with their ultracentrifugally purified material. The results obtained by the latter workers in such tests differed somewhat from those of Gratia and Paillot and indicated that both preparations contained serologically related material. Absorption tests, however, brought to light further significant facts. When the antiserum (produced in rabbits) to material from the blood of normal silkworms was absorbed with purified material from diseased blood, it was found that this antiserum gave no further precipitate on the addition of purified material from normal blood. On the other hand, when the antiserum to material from diseased blood was absorbed with purified material from normal blood, something remained in this serum, and the antiserum to material from diseased blood did give a precipitin reaction on the addition of material from diseased blood. In other words, antiserum to the purified material from diseased blood contains a serologically active component that is not present in the blood of normal silkworms.

It was also found that absorption of purified material from diseased blood with antiserum to purified material from normal blood removes considerable material but leaves a fraction having $s_{20}^{w} = 17$ S, which reacts strongly only with antiserum to material from diseased blood. This indicated that a considerable part of the purified material obtained from the blood of diseased silkworms consisted of a protein fraction that presumably represented the virus of jaundice.

At the close of World War II, when the scientific literature from Germany became available, it was revealed that some of the vagaries of the work done up to this time were being resolved by the work of Bergold and his associates. In 1943 Bergold published a review of their work up to that time. In this paper he expresses the belief that the vibrating granules (as described by Paillot and Gratia) seen upon the dissolution of the polyhedra with weak acids and alkalies are to be considered as virus aggregates. He also thought that the high-molecular protein making up these aggregates was probably identical with the virus protein. In other words, his work up to that time led him to believe that the polyhedron-virus protein may exist as virus molecules, and as polyhedral crystals made up of virus aggregates, and that the polyhedral protein itself was the cause of the disease.

In 1947 and 1948, however, Bergold informed the writer in correspondence that he intended to modify somewhat his conception of the virus. He contended that throughout all his preceding work, as well as that of other investigators, the actual virus was being overlooked. Further experimentation convinced him that the virus is present only to the extent of about 3 to 5 per cent inside the polyhedral bodies and that the polyhedral protein itself is distinct from the virus. He then proceeded to isolate the virus and found it to have a high particle-weight of about 916×10^6 (or of 299×10^6 when calculated from the dimensions of the particles as seen in electron micrographs). Electron micrographs showed the silkworm virus particles to have the shape of tiny rods with an average size of approximately 40 by 288 millimicrons. Similar bacterium-shaped virus particles were demonstrated in the case of the polyhedroses of larvae of the gypsy moth and the nun moth. The virus particles, as distinct from the polyhedral-protein, are infectious, are suspendible in water, have a high phosphorus content, and have a total nitrogen content of about 14 per cent. They are nucleoproteins (desoxyribonucleic acid), have a Svedberg sedimentation constant s_{20} of 1,871, a diffusion constant of 0.215×10^{-7}, a frictional ratio (f/f_0) of 1.51, and are infectious at dilutions of 10^{-11} grams of protein per larva.[1] They appear to lack a cell membrane and any definite internal structure, which would be points of difference between them and bacteria and rickettsiae. Their reaction to such agents as glycerin, alcohol, ether, and freezing, however, indicates that they have properties characteristic of living microorganisms. Other properties and reactions of the virus as determined during its association with the polyhedron will be mentioned in a later section.

[1] For a discussion of the chemical and physical procedures by which viruses in general are characterized, the reader is referred to an excellent treatment of the subject by Stanley and Lauffer (1948).

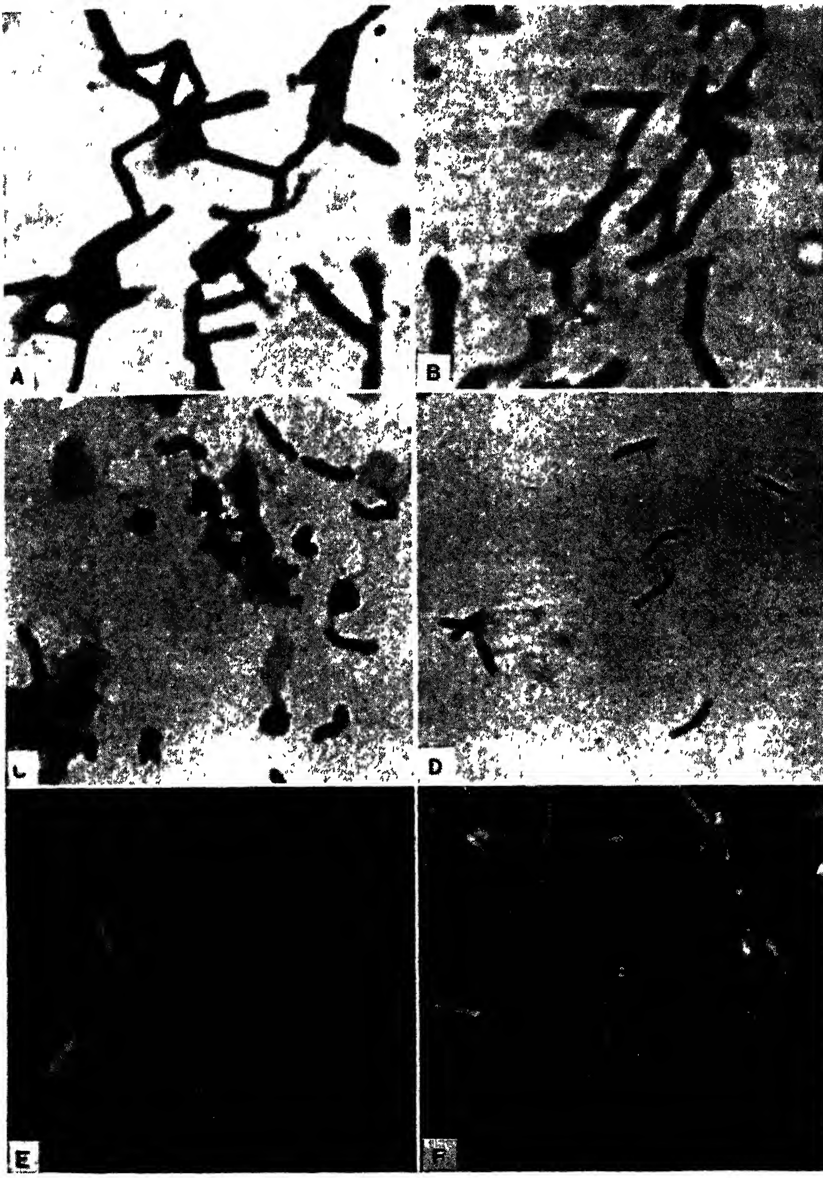


Fig. 132. Electron photomicrographs of the virus of silkworm jaundice taken at magnifications of about 30,000×. *A* and *B*. Mixtures of virus particles and bundles in water. *C* and *D*. Virus particles in 0.05*N* hydrochloric acid, showing evidence of spherical swellings at intervals along the particles. *E* and *F*. Preparations showing mostly individual rods. (*Courtesy of G. Bergold.*)

Particles of the size described as the virus by Bergold are not too small to be seen with a dark-field microscope. It is possible, therefore, that the "granules" seen and described by Paillot and others (and possibly the so-called "chlamydozoa" described by von Prowazek) were in fact the virus particles or bundles of virus particles.

Of considerable interest is the fact that the virus may occur in the form of a bundle of small rod-shaped particles which, at times, may be seen to separate from each other. In some preparations, especially after a treatment with acid, small spherical portions along the rods can be seen opposite each other when two or more particles are hanging together. This suggests that possibly the virus particles increase by splitting off in a type of "sideways multiplication." The bundle of silkworm virus contains probably only 2 to 4 single virus particles, and these particles seem to occur as a bundle less frequently than in the case of the virus particles of the polyhedrosis of the nun moth and that of the gypsy moth.

Although the results obtained by Bergold and those obtained by Glaser and Stanley (1943) agree in showing that the polyhedra contain only very small amounts of virus, they differ in certain other respects. Bergold found that the infectious agent is sedimented almost completely at about 10,000 r.p.m., whereas the protein isolated by Glaser and Stanley has a molecular weight of about 300,000 and would not sediment at 10,000 r.p.m. This means that the protein that Glaser and Stanley isolated from the blood of diseased silkworms is not identical with the infectious virus as isolated by Bergold.

It is difficult, at this point, to decide just what interpretation to give to the work of Yamafuji and Cho (1947) and Yamafuji and Yuki (1947) concerning the artificial production of the virus of silkworm jaundice. The conclusions of these Japanese investigators are based on the idea that undecomposed hydrogen peroxide promotes the polymerization of nucleoproteins to form virus. They found that jaundice could be produced if the insects were first held for 10 minutes at 45°C. and then fed hydroxylamine, which inhibits the catalase that ordinarily brings about the decomposition of hydrogen peroxide in animal tissues. When the hydrogen peroxide was thus prevented from decomposing, it presumably aided in bringing about the polymerization of the nucleoproteins and the formation of virus. In other words, according to Yamafuji and his coworkers, virus formation proceeds by the following steps: normal nucleoprotein → denatured nucleoprotein → polymerized nucleoprotein → virus molecules. These results would appear to need further confirmation and are perhaps open to interpretations other than those presented by the Japanese workers.

The only practical conclusion that appears safe at present concerning the general nature of the virus that causes jaundice in silkworms is that it

is an ultramicroscopic filterable agent having the chemical constituency of a nucleoprotein. In all probability it is identical to the rod-shaped bodies which have been demonstrated with the electron microscope.

Before discussing the nature of the relationship between the virus and the polyhedron, let us first consider the characteristics of the polyhedra themselves.

The Polyhedra of Silkworm Jaundice. The polyhedral bodies observable in the tissues and body fluids of silkworms stricken with jaundice are

Fig. 133. A view of the polyhedral bodies characteristic of silkworm jaundice, showing the distinct hexagonal shape of the polyhedra. *(Courtesy of R. W. Glaser.)*

similar to those found in insects suffering from other polyhedroses. They appear as highly refractive crystallike bodies occurring singly and frequently in pairs. Their size may vary from 0.5 to 15 microns in diameter, but they are usually uniformly 3 to 5 microns in diameter. Their shape also varies, but ordinarily five to eight faces (usually six) may be seen; most of the corners are sharp and angular, and the polyhedron never occurs as a true sphere. On focusing, the center of the polyhedron appears more dense than does the periphery. Concentric layers like those of an onion are frequently observed within the bodies, suggesting that they may "grow" by accretion. In glass-slide preparations, the polyhedra may be seen to crack and to fragment into a number of pieces when pressure is applied. Not infrequently, similar, but slower, fragmentation may be observed without the application of pressure. This almost classic conception as to the crystallike nature of polyhedra has not been held universally, even though much of the recent work supports it. Dikasova (1942), for instance, triturated polyhedra mechanically and found the material

not to be solid and dense but instead found it capable of being easily smeared or spread out. Incidentally, this Russian worker reported the characteristic presence of 15 or 20 small oval bodies contained within each polyhedron, apparently considering them of etiological significance.

With preparations from dead insects or from insects well along in the course of the disease, there is usually no difficulty in distinguishing the polyhedra. It has been estimated that 0.01 milliliter of blood from a silkworm 6 days after infection contains between 5 and 6 million polyhedral bodies. If an insect is examined in the early stages of infection, the few polyhedra present are frequently difficult to distinguish from other bodies that may be present. The inexperienced may confuse them with fat globules and urate or other crystals. In such cases it is often helpful to search out an infected cell in the nucleus of which the inclusions may be easily seen and recognized. Sometimes certain characteristics of the polyhedra may be used to differentiate them from other bodies. Thus fat droplets are perfectly spherical, are soluble in ether, and are stained with Sudan III. Polyhedra are never spherical in shape, are insoluble in ether, and do not stain with Sudan III. Urate crystals have an entirely different shape from that of polyhedra and often show radiating lines. When viewed with polarized light, polyhedra are not optically active, whereas most other crystals seen in insects are.

Polyhedra are heavier than water and for this reason usually settle to the bottom of a wet-mount preparation. They are insoluble in hot or cold water, alcohol, chloroform, ether, or xylol. They are soluble in acids or in alkalies, especially when boiled in them or when allowed to stand in them for some time. When subjected to the dissolving action of alkalies or acids, the polyhedra become markedly swollen, and a granular mass becomes visible in their interior. The optimum dissolutions are obtained in ranges of pH 1.0 to 0.5, and pH 10.8 to 11. Following the proper procedures, dissolved polyhedra can be recrystallized. Such recrystallized polyhedra dissolve more easily and more quickly in weak alkalies than did the original polyhedra. Also the recrystallized polyhedron appears to be devoid of the "enveloping membrane" that is ordinarily present at the surface of the polyhedron. In 30 per cent trichloroacetic acid the polyhedra do not dissolve but flow into one another, forming a white coagulated film that is irreversibly denatured. That they contain protein is grossly indicated by the fact that picric acid stains them yellow. That they contain no fat is indicated by the fact that, in addition to their not staining with Sudan III, they also do not blacken with osmic acid. They do not stain readily with most aniline dyes, but they can be thus stained if the preparation is heated or if a mordant is used.

It has been determined (Bergold and Schramm, 1942; Bergold and

Hengstenberg, 1942; Bergold and Brill, 1942; Bergold, 1943, 1948*b*) that the polyhedral bodies are protein crystals that dissolve in weak alkali (*e.g.*, 0.006*M* Na_2CO_3) to a very homogeneous protein the principal component of which in turn has a molecular weight of about 378,000 and a diameter of about 10 millimicrons. (The molecular weight of the split components is about 60,500.) The Svedberg sedimentation constant s_{20} of the principal component is 12.85; that of the split components is 3.16.

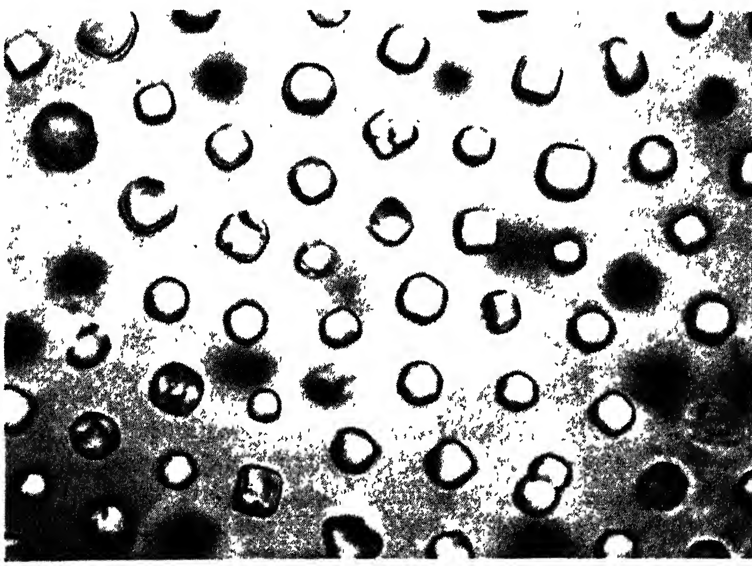


Fig. 134. Photograph of suspension of polyhedral bodies present in silkworm jaundice as seen with the high power of an ordinary light microscope. (*Courtesy of R. W. Glaser.*)

The polyhedra appear to consist of about 95 per cent of this protein, and 5 per cent virus protein. According to Bergold, 82 per cent of the weight of the polyhedra crystals is polyhedral protein. Unlike the virus protein, the polyhedral protein will not dissolve in water, has a total nitrogen content of about 15 per cent and a low phosphorus content, and has been shown to be incapable of bringing about an infection.

Desnuelle and his coworkers (1943) determined the phosphorus, sulfur, and amino acid content of the polyhedral protein which they assumed, on the basis of Bergold's earlier work, to be identical with the polyhedral virus. Since it is now known that the two are not identical, their results may be taken as applying more to the polyhedral protein than to the virus protein. They ascertained the molecular weight of the protein to be in the vicinity of 195,000, which corresponds to the presence of 8 atoms of

phosphorus and 4 molecules of cystine per molecule of protein. The amino acid content consisted of the following percentages: histidine 2.5, arginine 5.6, lysine 10.6, tyrosine 9.6, phenylalanine 6.7, tryptophane 3.3, cystine 0.52, methionine 3.3, and alanine 4.4. Desnuelle and Chang (1945) produced a modification of the protein which differed from the normal protein only in that its amino groups were all acetylated. If such were accomplished for the virus protein, which these workers thought they did, it would be exceedingly interesting to observe the physiological activity of the material. Desnuelle and Chang obtained another type of polyhedral protein by treating a suspension of the original protein in anhydrous methanol with dry hydrochloride gas. All the carboxyl groups of the original protein became methyl esters. The free basic groups became hydrochlorides. Then, by an analysis of the chlorine content of the modified protein, the number of free basic groups carried on the protein by arginine, histidine, and lysine could be determined. They report the protein molecule to have 111 esterifiable groups and 67 amide groups. When certified pure suspensions of the virus protein itself are obtained, it will be interesting to make a comparison of these data.

Iron (about 0.5 per cent) has also been reported in polyhedra, and Manunta (1940), on the basis of a positive Molisch reaction, believes that the presence of carbohydrates is indicated. This last observation has not been confirmed, and perhaps needs reinterpretation.

The Relation of the Polyhedra to the Virus of Jaundice. As we have already pointed out, although Bolle (1898) believed that the polyhedra of jaundice in silkworms represented the sporulated form of a sporozoan parasite, contemporaries of his were concerned with the crystalline nature of these bodies. When it was discovered that the disease could be initiated in the absence of the polyhedra, the conclusion was made that these visible inclusions were merely by-products of the disease and had no relation to the virus itself. Especially was this the case when Glaser (1927) reported that he was able to free the polyhedra completely from the virus by repeated washing and centrifuging. Other investigators, however, had not been able to obtain comparable results, and Glaser himself, on the basis of further work (Glaser and Lacaillade, 1934), later altered his opinion. Aoki and Chigasaki (1921) with repeated washings had been unable to free the polyhedra entirely of their virus activity, and on the basis of the results of serological tests they maintained that the polyhedra are unrelated to the protoplasm of the cells in which they are formed. Glaser and Lacaillade found that, when freshly drawn jaundice blood, containing polyhedral bodies, was immediately centrifuged, the supernatant fluid free from the inclusions was highly infectious. If the blood was allowed to stand for some time, the polyhedra settled and,

in the upper layer of the fluid, which was free of them, no virus was found. Repeated washings removed considerable amounts of the virus from the polyhedra, but it appeared impossible to remove it entirely by this method. A similar result was obtained when the pH of the wash water was varied. Iodine solution, 80 and 95 per cent alcohol, and 5 per cent potassium dichromate, tried for varying periods of time, and 5 per cent phenol for 1 hour, did not sterilize the polyhedra. Heat, however, was effective in rendering inactive the virus associated with polyhedra. A temperature of 60°C. for 30 minutes did not always sterilize the polyhedra, but this was accomplished by heating them for 30 minutes at 100°C. When the sterilized polyhedra were brought in contact with active virus, this agent apparently became associated with them again to some extent. These results indicated that the polyhedra are physical carriers of the virus, but it was not demonstrated whether the active virus was present or absent within the polyhedral bodies. Earlier, Komárek and Breindl (1924) had expressed the belief that the causative agent was carried in the interior of the polyhedra, but Glaser and Lacaillade were of the opinion that the virus was only adsorbed on the surface of the inclusions, which were merely by-products of the infection.

The latter view was challenged in 1939 when Gratia and Paillot published the claim that the polyhedral bodies are crystalline agglomerates of the virus. Furthermore, they reported that the characteristic minute granules or elementary bodies, which they believe represent the virus, have antigenic properties identical to those of the polyhedra. The antiserum prepared against the polyhedra, and that prepared against the virus particles, cause the agglutination not only of their own antigens but of the heterologous antigens as well, up to a dilution of at least 1 : 3,000. These same serums, however, were without effect on the constituents of normal tissues. Inversely, antiserum against normal tissues does not affect either the virus particles or the polyhedra of diseased silkworms. Moreover the polyhedra of silkworm jaundice are antigenically distinct from those of the polyhedrosis of the cutworm *Euxoa segetum* Schiff. Thus these workers conclude that the small virus particles and their supposed crystalline agglomerates, the polyhedra, represent a specific antigen distinct from the tissues that harbor them and that, therefore, both appear to be of a foreign parasitic nature. Presumably they occupy a place intermediate between the "elementary bodies" that form amorphous agglomerates such as those of vaccinia, and the artificially crystallized proteins such as those of certain plant viruses.

Additional evidence to support their idea was presented by Paillot and Gratia (1939) in a second paper. According to this report, if a small amount of 0.5 per cent sodium hydroxide is introduced into an emulsion

of polyhedra under an ordinary microscope, they appear to be completely dissolved. If the same operation is repeated under a dark-field microscope, the polyhedra are seen to lose their luminous quality and their firmness of outline, but they remain still faintly visible as dull, spread-out, spherical masses in the midst of which are large numbers of minute granules animated by Brownian movement. These granules appear entirely comparable with the "virus" particles seen in virulent blood. These and other observations caused the authors to reaffirm their conclusion that the polyhedron is an agglomerate of elementary particles oriented according to a sharply defined, crystalline form, but that besides this it contains other substances that form the "shell" of the polyhedron.

Incidentally, Paillot and Gratia paid considerable attention to the crystalline nature of the polyhedra and report that when washed in distilled water and dried, the perfectly hexagonal contour of these bodies may be confirmed. With the polarizing microscope the polyhedra show no birefringence. To these workers it seemed that these facts pleaded the crystalline nature of the polyhedra, which appear to be of the cubic system and probably of the rhombic dodecahedra form.

The conclusions of these French workers have not, however, been entirely accepted. Glaser and Stanley (1943) studied the biochemistry of the virus and of the polyhedra of silkworm jaundice and decided that virus material was occluded within these bodies but that no great concentration of virus activity is found within them. The American workers were not ready to accept Gratia and Paillot's idea that the polyhedra were crystalline agglomerates of the virus. Although the inclusions were active, they were no more active on the nitrogen basis than the diseased blood free of polyhedra. Blood was inactive after being subjected to hydrogen-ion concentrations more acid than pH 5. The polyhedral bodies, on the other hand, possessed activity even after standing at pH 2 for 24 hours. This indicates that virus is contained within the bodies which protect it from the action of the acid. Similar results were obtained with 1 per cent sodium dodecyl sulfate and with antiformin-formalin.

Glaser and Stanley found that a chemical analysis of supposed virus material showed a composition differing somewhat from that of the polyhedra, although both were compatible with a nucleoprotein composition. When examined by means of an electron microscope, the polyhedra, washed six times with water, showed much mucilaginous material adhering to the surfaces (Fig. 135), suggesting that material is extruded from the inclusions despite repeated washings. That the polyhedra represent crystallized virus, however, appears untenable according to Glaser and Stanley. They consider them to be by-products of the disease and believe that, while

being formed in the nucleus of an infected cell, the polyhedra fortuitously occlude virus material within them.

Recent developments tend to confirm much of Glaser and Stanley's concept as to the relation between the virus and the polyhedron. Bergold (1943) originally believed that the polyhedron consists of virus aggregates, as did Paillot and Gratia. He concluded that the polyhedral protein is probably identical with the infectious virus protein. Upon the basis of new experiments and upon rechecking the results he had obtained, Bergold,

Fig. 135. Washed polyhedral bodies obtained from jaundice-diseased silkworms, showing mucilaginous material adhering to the surfaces. Electron microscope magnification of about 7,600×. (*From Glaser and Stanley, 1943.*)

with scientific honesty, changed his opinion in a manner that conforms more closely, in some respects, to that held by Glaser and Stanley. He became convinced that the polyhedral protein, which makes up about 95 per cent of the polyhedron, and the infectious virus protein are not identical. The virus constitutes only about 3 to 5 per cent of the content of the polyhedron. As has already been mentioned, the virus appears to have the form of bacteriumlike rods about 40 by 288 millimicrons in size, and it is apparently incorporated within the structure of the polyhedron. When the polyhedra are dissolved in a weak solution of sodium carbonate, the virus may be released in such a way as to leave clear areas or holes in the polyhedral mass—additional evidence that the virus is an entity distinct from the polyhedron itself. Also of interest is the fact that the polyhedral protein and the virus protein can be split into small parts that are capable of aggregating again to form high-molecular entities.

According to Bergold, there nevertheless appears to be a definite serological relationship between the polyhedral protein and the virus protein. Theoretical explanations for this include the following pos-

sibilities: (1) The polyhedral protein may be a component part of the virus but lacking the nucleic acid; *i.e.*, it is the same as virus protein minus the nucleic acid. (2) The polyhedral protein is a decomposition product of the virus. (3) The polyhedral protein is a host-reaction product that crystallizes about the virus in the form of a polyhedron and partly acquires a common antigenicity. The polyhedral protein might be comparable to the "soluble antigen" of other viruses like that of smallpox. It is of interest to note that the elementary bodies of the latter can be split into two fractions: one distinct nucleoprotein and another nucleoprotein serologically related to it.

It should be remembered that, in the past, most of the determinations with respect to the properties of the virus of silkworm jaundice have been made using suspensions containing polyhedra. Since the virus appears to be incorporated within the polyhedral bodies, the results obtained from these determinations must take into account the protective properties of the polyhedra. Nevertheless interesting results have been obtained following the treatment of such suspensions with various chemicals and reagents. Bergold (1943), for example, found that polyhedra suspensions remain infectious after 15 minutes' treatment with the following: acetone, ether, 2.5 to 30 per cent formaldehyde, 5 per cent carbolic acid, 5 per cent mercuric chloride, 70 per cent alcohol, and a 1 : 1 solution of 96 per cent alcohol and 1 : 1,000 mercuric chloride. Furthermore polyhedra remain infectious after being stored for 22 months in a 1 : 1 solution of 1 per cent sodium chloride and glycerin, or in 1 per cent toluene, or in 0.1 per cent zepharol, at 4°C. Polyhedra also remain infectious after having been exposed in a thin layer to the rays of the sun for from 2 to 10 hours; after having been dried out for 5 hours in a high vacuum of 10^{-4} millimeter of mercury; and after having been stored for 22 months in the tissues of larvae in the process of putrefaction, or in 0.85 per cent sodium chloride at 4°C. On the other hand, the infectiousness is greatly weakened when polyhedra, suspended in water, are boiled for a short period of time. The virus activity is entirely destroyed if they are boiled for 10 minutes or if they are treated for 15 minutes with trichloroacetic acid.

We have subjected the reader to a rather detailed summary of the results of the principal investigations dealing with the nature of the silkworm virus and the associated polyhedra not only because the subject is still somewhat in a state of uncertainty but because it has almost become the classical polyhedrosis for investigation. The recent findings of Bergold and his group appear to be very convincing. Many of the results obtained by Paillot and Gratia need explanation before they can be correlated with those obtained by Bergold. Eventually, perhaps, the numerous conflicting viewpoints will be reconciled by further penetrating research.

Until then we are forced to keep the various possibilities in mind and to reserve final judgment until all the evidence is in.

Pathology of Jaundice in Silkworms. The natural course of jaundice in the silkworm begins with the ingestion of infectious material (polyhedra or free virus) into the alimentary tract of the animal. It has been assumed that the alkaline reaction of the silkworm gut, as well as certain enzymes present there, dissolves the polyhedra, liberating the virus which then passes through the intestinal wall into the body of the insect. (It appears that the minimum infectious dose of polyhedra-virus protein capable of bringing about oral infection varies according to the degree with which virus bundles are dissociated. The following values have been reported in terms of grams of protein per larva: 1.5×10^{-10} gram [Bergold and Schramm, 1942], 4×10^{-13} gram [Bergold, 1948*a*], and 1.0×10^{-11} gram [Bergold, 1948*b*]). Once through the intestinal wall, the virus circulates throughout the body cavity and invades the cells of the susceptible tissues.

Jaundice-diseased silkworms usually begin to assume a blotchy, yellowish appearance in from 4 to 7 days and die in from 10 to 14, or as long as 18 days after infection. Just before death most of the internal tissues disintegrate, and this dissolution becomes complete a short while after death. In such a condition it is almost impossible to remove the larvae without disrupting them and liberating the dark viscous liquid contents. If one makes a microscopic examination of this liquid material, it is seen to be filled with polyhedra floating free and enclosed within the cells of certain tissue fragments. An accurate idea as to the histopathology of the disease cannot, however, be obtained by the examination of such disintegrated specimens. Instead living diseased material should be used, and examinations should be begun in the early stages of infection and followed through, using larvae progressively further along with the disease. Examination of the blood as well as of tissue fragments can be made from smear preparations; but, for most of the detailed histopathology, sectioned tissues should be used.

Descriptions of the pathological changes occurring in jaundiced silkworms have been presented by Glaser (1927) and by Paillot (1930*b*, 1933), and the following account is based upon their observations.

Although some authors maintain that the hypodermis and fat tissue may show polyhedra before the blood cells do, it has been established that one of the earliest indications that the disease is present may be conveniently gained by examining the blood. We shall therefore consider first the pathology observable in this part of the insect. The principal types of blood cells or hemocytes in normal silkworms are: leucocytes (40 to 50 per cent of the blood cells), proleucocytes (25 to 30 per cent), lymphocytes (10 to 15 per cent) (proleucocytes are commonly included

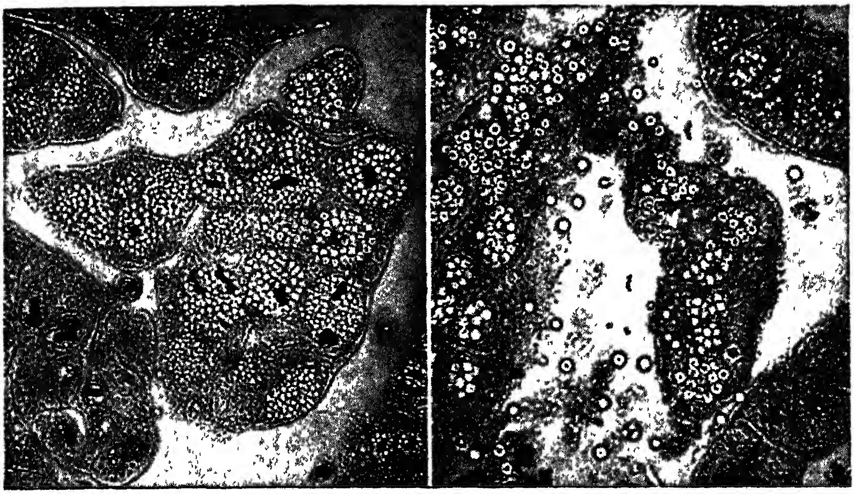
with the lymphocytes), spherule cells (10 to 15 per cent), and a very small number of oenocytoids. (The reader will find a description of these cells in Chap. 7.) In virus-diseased silkworms, the leucocytes and lymphocytes are the blood cells in the nuclei of which one may observe the development of polyhedra. When the nuclei of these cells (polyhedra are never found within the nuclei of the spherule cells or the oenocytoids) are filled with the inclusions, it is certain that the insect will succumb to the disease. Sometimes a very few polyhedra are seen in the nucleus of an occasional leucocyte, in which case one cannot be certain that a frank infection will follow. Occasionally the bodies are found in the cytoplasm; it is assumed that these have been phagocytosed. The origin of the inclusions in the nuclei of the blood cells is preceded by a concentration of the nuclear substance and the formation of a central denser mass around which refractive granules appear. These granules gradually develop into polyhedra and eventually completely fill the nucleus. The cells finally become disorganized and liberate the polyhedra, which float free in the hemolymph. The milky appearance of the blood of heavily diseased caterpillars is due to the presence of these polyhedra, together with the fat droplets being liberated from the disintegrating fat body. Thus, by frequent examinations of the blood, it is possible to obtain a fairly clear idea as to whether or not the insect is affected by a polyhedrosis, and an estimate as to the extent of the infection, as well as a general prognosis, may also be gained.

The other tissues of the infected silkworm that show a characteristic pathology and the presence of polyhedra in the nuclei of the cells are those of the hypodermis, fat body, genital capsule, and tracheal matrix. Polyhedra have sometimes been reported to occur within the nuclei of tissue cells other than those mentioned, but not with any degree of regularity. Under certain conditions, for example, the epithelial cells of the midintestine may show pathological changes. This occurs when *Bacillus bombycis* is present, since this bacterium seems to condition the epithelial cells for virus infection, and vice versa. This and similar possibilities must be kept in mind when studying the histopathology of virus diseases in insects.

At the beginning of jaundice infection, tiny granules appear in the cytoplasm of the affected cell. By the second day certain changes may be seen taking place in the nucleus. Normally the nucleus of a fat cell, for example, contains small round grains of chromatin distributed throughout it, and the nucleoli are large and numerous (usually 10 or more). In the diseased cell, the chromatin grains and the nucleoli fuse, forming a large, dense, highly chromatophilic mass. Within this mass appear minute refractive bodies that probably originate from the chromatin grains. These bodies, which do not take stains, gather at the periphery of the chromatophilic mass, which is dotted with fuchsinophilic granules,

and form a ring about it. According to Paillot, the nucleus contains a liquid formed apparently by the liquefaction of some of the chromatin material by the virus. In this liquid the polyhedra arise as very small individuals. They increase in size, becoming more refractive; do not take stains; and finally fill the entire nucleus, which becomes greatly hypertrophied.

Within a single nucleus the polyhedra are usually all of the same



A *B*

Fig. 136. Polyhedral bodies of silkworm jaundice in the fat tissue of a diseased silkworm, as seen with the high power of an ordinary light microscope. *A*. The nuclei of the fat cells are hypertrophied and filled with polyhedra. Condensation of chromatin has occurred in some nuclei; in others the chromatin has completely disappeared. *B*. A later stage of the infection. Lysis of the tissue is beginning, together with the liberation of the polyhedra from the disintegrating cells. *(Courtesy of R. W. Glaser.)*

general stage of development, although, as Bergold (1943) has demonstrated, this is not always the case. Great differences of development do occur, however, between the inclusions of different nuclei. The small formative polyhedra are more nearly round than are the larger ones; but as they increase in size, they become closely packed together. It has been assumed by some that, by their pressing upon one another, the characteristic faces and angles of the polyhedron are formed. That the faces are formed in this manner has been denied by several workers. It has also been noticed that in tissue cultures the inclusions may be of polyhedral shape even when they are not crowded together. In this connection, it is interesting to note that in the fat tissue of infected silkworms, a nucleus showing tetrahedra in the place of the normal rhombododecahedra will

contain *only* tetrahedra, whereas the adjoining nuclei will show *only* the normal hexagonal forms (Bergold, 1943). As the polyhedral bodies increase in size and number, the tiny dispersed granules, as well as the remainder of the chromatin mass, disappear, leaving only the polyhedra enclosed by the nuclear membrane. Paillot believed the number of granules appear to become fewer as the polyhedra increase in size and that

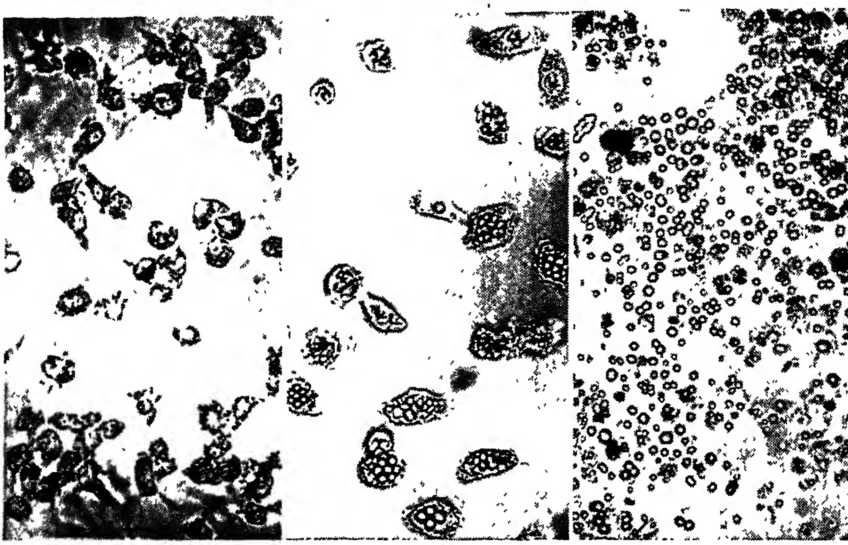


Fig. 137. Photographs showing the production of polyhedral bodies in silkworm-tissue cultures inoculated with the virus of silkworm jaundice. *A*. Normal 6-day-old tissue culture. *B*. A culture 5 days after infection with the virus. *C*. A culture 8 days after infection with virus. The infected cells have disintegrated, liberating the polyhedra in large numbers. (*From Trager, 1935.*)

this was another indication that the polyhedra are actually agglomerates of the elementary virus particles. During this time, the cytoplasm of the infected cell undergoes some changes. Particularly noticeable is the disintegration of the filamentous mitochondria, which become small dispersed granules. When the nucleus is completely destroyed the entire cell disintegrates and the polyhedra are liberated into the body cavity of the insect. Coincident with the disintegration of the cells, but apparently independent of it, is the lysis of all the affected tissues. Only after the insect dies, or is moribund, are any significant changes apparent in the cells of the muscles, Malpighian tubes, salivary glands, nerves, and other tissues not harboring polyhedra.

Many of the pathological changes described here have been demonstrated in a tissue-culture medium composed of certain cells from the

gonads of female silkworms. In such a medium, Trager (1935) found that the number of cells showing well-formed polyhedra varied with the condition of the tissue culture at the time of infection and during the next few days. The healthiest cells give the best and most rapid polyhedra formation. Polyhedra frequently begin to appear within 24 hours after infection and are present in most of the cells within 48 hours. During subsequent days the inclusions increase in size and number. About a week after the culture has been infected, the cells begin to die. The cytoplasm becomes dense and granular, and amoeboid movement ceases. Some of the dead cells burst and liberate the contained polyhedra; this process continues until the culture has degenerated into a mass of tissue debris and large numbers of free polyhedra. In some cultures the cells die but the polyhedra are retained within the dead cells.

Transmission, Immunity, and Control. In nurseries the virus of jaundice may be transmitted from insect to insect in several ways, but the exact manner of the majority of the transmissions has not been determined. Most of it probably occurs during contact between individual silkworms or by the ingestion of virus-contaminated food. Interestingly enough, however, the experimental feeding of contaminated food is by no means a certain method of producing the disease—at least when compared with the results obtained by direct inoculation into the body cavity, which is nearly always successful except in immune larvae. Fecal contamination may be a factor, since it is known that the intestinal contents of infected silkworms may be infectious. The infection may also be spread when the integument of a larva is broken so as to allow the infected blood to escape onto the screens and other rearing equipment.

Great care should be taken in removing the sick and dead caterpillars, since their skin is so fragile that rough treatment will cause it to break and liberate the infectious contents. One method of separating healthy larvae from diseased ones has been presented by Paillot: A large sheet of thin paper, perforated with holes, is placed on the tray containing the silkworms. On top of the paper are placed fresh mulberry leaves. The active healthy caterpillars will then make their way up through the holes to get at the fresh leaves. When all the caterpillars are on the paper, it is lifted off and removed to another breeding tray. The diseased insects are thus left behind and are removed and destroyed. Incidentally, great care should also be taken when cleaning the contaminated trays, since any dust raised not only contains the virus of jaundice but in itself may cause a type of amicrobic dysentery (see Chap. 3).

The caterpillars appear to be much more sensitive to infection during the periods of molting. Other predisposing factors include high temperatures and humidities. The infection in silkworm nurseries is greatly

intensified by a rise in temperature. Paillot found that silkworms inoculated with the blood of sick insects and placed in a room with the temperature varying between 16 and 17°C. showed no symptoms of jaundice 15 days after the inoculation. Caterpillars inoculated in the same fashion and held at a temperature of 25°C. were in the advanced stages of the disease 6 days after the inoculation.

There have been several reports (Rebouillon, 1925; Paillot, 1926*b*, 1930*b*) on the detection of the virus and the polyhedra of jaundice in adult moths of the silkworm. Paillot also claimed to be able to detect virus-infected eggs. He inoculated a healthy adult moth, before it began laying eggs, with a drop of blood from a diseased larva. The eggs from this moth were then examined with a dark-field microscope. According to Paillot, the same etiological granules found in the infected blood were present in the egg. Paillot (1930*b*) then makes the cryptic statement that a certain number of moths may die of the infection before the appearance of "the bodies" in the tissues. Although transmission of the virus of jaundice through the egg is thus indicated, it would appear that more informative and conclusive results are to be desired. There is evidence that the virus survives on the surface of the egg, and this type of transmission should be clearly differentiated from that in which the virus may be present within the egg. It is not at all clear whether the latter can take place. It has been demonstrated that disease-free larvae can be reared from contaminated eggs the surfaces of which have been sterilized by some suitable procedure such as washing them for 15 minutes in 30 per cent trichloroacetic acid and then in water. In any case, the elimination of diseased moths by the ultramicroscopic examination of either their tissues or their eggs would probably not be a very practical procedure for sericulturists, nor would it be so simple or so certain as the comparable method used by Pasteur for the detection of pebrine-infected moths and eggs (see page 601).

There are indications that a certain number of caterpillars are immune to the virus of jaundice and, despite exposure to or infection by the virus, are able to complete their metamorphosis into moths. Whether this immunity is the normal variety or whether it depends on previous contact of some kind with the virus is not clear.

Definite and specific measures for the control of jaundice in silkworms have never been formulated and put to general use. If transmission by way of the egg is common, control measures, to be effective, would have to be directed toward the eradication of diseased eggs. In addition, strict sanitary rearing methods are required: the disease cannot occur if the virus is not present. As with the other diseases of the silkworm,

careful supervision of such factors as temperature, humidity, food, and ventilation must be maintained.

Speyer (1925) found that, after the administration of sublethal doses of arsenic compounds to silkworms suffering from polyhedral infection, a higher percentage of the caterpillars pupated than would otherwise be the case.

Susceptibility of Other Insects. The susceptibility of insects other than the silkworm, *Bombyx mori* (Linn.), to the virus of jaundice has been the subject of contradictory evidence. As has been mentioned, some of the earlier workers on the virus diseases of insects claim to have infected not only other Lepidoptera with the virus of silkworm jaundice but even a beetle (*Dermestes lardarius* Linn.). The larvae of certain other beetles, *e.g.*, *Leptinotarsa decemlineata* (Say), are definitely known to be insusceptible. All these early claims of interspecies susceptibility are highly questionable, and the presumed susceptibilities should be retested.

Careful experiments by Glaser (1927) failed to show any susceptibility to the silkworm virus on the part of the tent caterpillar, *Malacosoma americana* (Fabr.), which is highly susceptible to a virus of its own. Bergold (1943), however, states that the larvae of *Porthetria dispar* (Linn.), *Lymantria monacha* Linn., and *Dendrolimus pini* Linn. are experimentally at least partly susceptible to the virus of silkworm jaundice. The polyhedra found in the larvae that died in these tests in most cases had the form typical for the host animal. Although these results indicate that between rather closely related species the host specificity of the silkworm virus probably is not so strict as has been supposed, it is probably safest to withhold judgment on this matter for the time being. Absolute assurance as to the impossibility of incidental infection or of contamination with the virus specific for the host concerned is required before any generalization can be safely made. The same applies to experiments in which silkworm larvae, although resistant to the viruses of *L. monacha* and *D. pini*, appear to be susceptible to the virus of *P. dispar* administered both orally and intralymphally.

Polyhedrosis (*Wipfelkrankheit*) of the Nun-moth Caterpillar

In 1889 and in 1892, there occurred among caterpillars of the nun moth, *Lymantria monacha* Linn., which were destroying large sections of the spruce forests of central Europe, a peculiar disease that killed off enormous numbers of the insect. The infected caterpillars showed a loss of appetite and became very flaccid; and if they were disturbed shortly before or after death, the broken skin liberated a fluid of disintegrating internal tissues. The length of time from oral infection to death averaged from

13 to 15 days. Before dying, the larvae usually migrated to the tops of the trees where they could be seen hanging by their prolegs in large numbers. This characteristic of proceeding to the tops (*Wipfeln*) of the trees caused the disease to be known in Germany by the name *Wipfelkrankheit*, or *Wipfelsucht*, although other names have been employed occasionally. It might be mentioned, however, that the infected adults have no inclination to migrate to the treetops. According to Růžička (1932), females infected with *Wipfelkrankheit* are unable to get very far up on the tree to lay their eggs; they even oviposit on the ground litter. Thus a concentration of eggs near the ground indicates the occurrence of the disease. (Healthy females oviposit on any part of the tree trunk.) As is true in the case of most other polyhedroses, if infection takes place in the last larval instar, the insect may pupate, and die in the pupal stage, or it may survive the pupal stage and become an adult, retaining the polyhedra in its tissues (Heidenreich, 1940). Except as just indicated, the disease appears to make little further progress in the adult which, if its vital tissues have not been injured, is able to survive in an almost normal fashion. In his experiments, Bergold (1943) found 15.6 per cent of nun-moth larvae infected in the last and next to the last instars to survive to the pupal or to the adult stage. Usually the disease has its effect during the later instars, but in recent years it has become evident that the infection may be responsible for more mortality among young individuals than had hitherto been assumed.

Causative Agent. The cause of *Wipfelkrankheit* was at first believed to be bacterial in nature. Hofmann (1891) designated as *Bacillus* "B" the organism he studied. Von Tubeuf (1892*a*,*b*) isolated a bacterium which he named *Bacterium monachae*. Tangl's (1893) bacteriological contributions did not clarify matters any. In 1894, Eckstein isolated a sporeforming bacterium, *Bacillus monachae*, which he considered to be the same as *Bacterium monachae* and *Bacillus* "B." In 1911, von Tubeuf changed his opinion somewhat and assumed that *Wipfelkrankheit* develops when a variety of intestinal bacteria become dominant. In the meantime, polyhedral bodies had been observed in the tissues of the diseased caterpillars. As in the case of silkworm jaundice, these bodies were considered by some (Wahl, 1909–1912) as reaction products of the disease, by others (Escherich and Miyajima, 1911; Komárek and Breindl, 1924; Růžička, 1925) as carriers of the virus, and by still others (Wolff, 1910; Knoche, 1912) as a form of the causative agent itself. Supporters of the latter belief assumed that the polyhedra were sporozoan parasites or their cysts, and some authors considered it to be the same species (*Chlamydozoon prowazeki*) which Wolff had ascribed to the polyhedrosis of *Bupalus piniarius* Linn. The belief in the protozoan or protozoanlike cause of the disease was

largely overcome with the finding that this agent, as well as that of silkworm jaundice, was filterable and ultramicroscopic. The ideas that the polyhedra were simply by-products of the infection and that they were etiologically unrelated to the cause of the disease gained credence with a series of publications on the subject by Wahl (1909, 1910, 1911, 1912). At the present time it is known that *Wipfelkrankheit,* which is similar to the type of disease known in America as "wilt," is caused by an ultramicroscopic virus and that, like silkworm jaundice, the relation of the virus to the polyhedral bodies is an intimate but not necessarily dependent one.

To the *Wipfelkrankheit* or nun-moth wilt virus Holmes (1948) has given the name *Borrelina efficiens.* In so doing he makes no mention of the name *Crystalloplasma monachae* which Prell (1926) gave to the granules he saw within the polyhedra characteristic of the disease. Since the name given by Paillot to the granules characteristic of silkworm jaundice has been retained for what we now know to be the virus, the point might be raised that the same should be done in the case of Prell's granules. However, it could also be argued that, since Prell apparently considered the granules to represent nuclei of an encysted organism of some sort, the name he proposed is invalid. Until the situation can be further clarified, it is perhaps most convenient to accept the name *Borrelina efficiens,* although unfortunately Holmes, in giving this name to the virus, avoided any mention as to his concept of the exact nature of the agent, which has been more completely described by Bergold.

Characteristics of the Virus and the Polyhedra of *Wipfelkrankheit.* The virus of *Wipfelkrankheit* has not been studied to so great an extent as has that of silkworm jaundice; hence not as many of its individual characteristics are known. The virus appears to be somewhat larger than the virus of silkworm jaundice. It is less slender than the silkworm virus and more nearly oval in shape. The arrangement of the relatively thick virus particles into bundles of two members each is frequently observed. As far as is known, the virus of *Wipfelkrankheit* has the same general physical and chemical properties as does the virus of silkworm jaundice.

The polyhedra are more or less triangular in shape and average a little over 2.5 microns in diameter but may range up to 10 microns. Wahl reported the presence of polyhedra in newly hatched larvae of the nun moth, in the pupae and in the adult moths.

Infectious material has been found to retain its virulence for 3 years when held in a dry state. Held moist, in glycerin, it retains its virulence for at least 5 days. The virus also withstands putrefaction. In nature the virus is probably aided in its survival by the protective quality of the polyhedra, which become widely distributed. That polyhedra may occur

in considerable numbers in the soil has been shown by Komárek and Breindl (1924), who isolated them from this source by centrifuged washings.

Transmission of the virus is believed to take place largely through the ingestion of infectious material along with the food. Cannibalism may also play an important role, especially when eggs hatch at different times and the newly hatched larvae can feed on the bodies of the larvae that died of the disease during the preceding generation. Dissemination of the

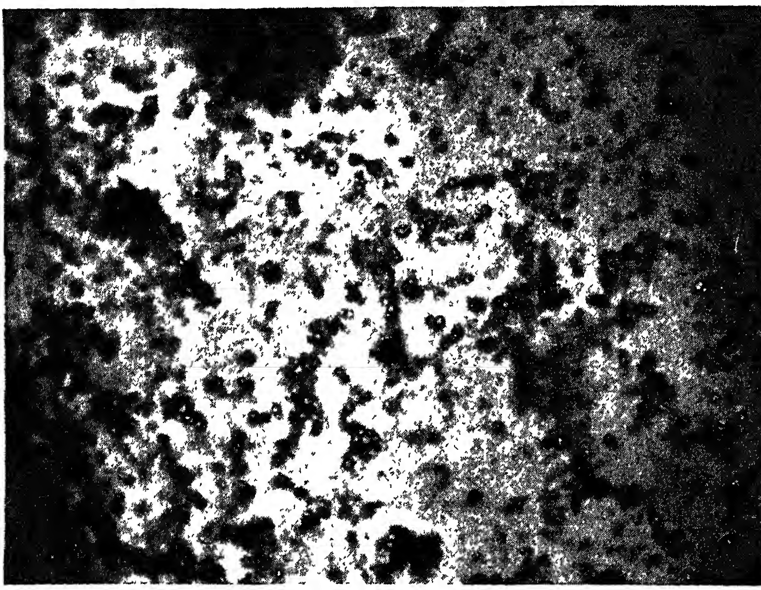


Fig. 138. Polyhedral bodies characteristic of nun-moth larvae suffering from *Wipfelkrankheit*, as seen at a magnification of about 500×. (*Courtesy of R. W. Glaser.*)

virus also takes place when the skins of the dead insects hanging in the treetops burst, distributing the virus and polyhedra about. Furthermore the polyhedra apparently overwinter in the soil and the forest litter which, scattered about by the winds and air currents, probably aids in the dissemination of the virus. Růžička (1925) holds to the theory that the "Chlamydozoa" cause infection in the insect during the larval stage, then hibernate in the polyhedral bodies, which disintegrate in the forest litter, freeing the organisms that enter the uninfected larvae through the spiracles. Individual insects need to ingest a very small amount of infectious material to become infected. The minimum infectious dose for oral infection is in the neighborhood of 2.5×10^{-15} gram.

According to Bergold (1943), larvae of *Porthetria dispar* (Linn.) and *Dendrolimus pini* Linn., but not those of *Bombyx mori* (Linn.), are partly

susceptible to the *Wipfelkrankheit* virus. Nun-moth larvae appear to be susceptible to the viruses that ordinarily affect all these insects.

The gross pathology and the histopathology of *Wipfelkrankheit* appear to be similar to those characteristic of most of the other polyhedroses. Minor variations have been cited by Breindl (1938) and by Heidenreich (1939).

Economic Aspects. Nearly all European authorities have emphasized the great economic importance of *Wipfelkrankheit* in aiding in the control of the nun moth in forests of central Europe. The disease becomes epizootic after the population of the pest has been building up for 2 or 3 years. In western Czechoslovakia, between 1917 and 1927, the disease gradually made its appearance among the nun-moth population and spread rather extensively, but, in general, it appeared too late in the season to prevent the greater part of the damage brought about by the insects (Komárek, 1931). Greater benefits could perhaps be obtained if methods were known whereby epizootics could be initiated earlier in the season than the disease occurs naturally.

Considerable effort has been extended toward artificially introducing the polyhedrosis among larvae of the nun moth in the forests of central Europe. Růžička (1924), for example, carried out a program of distributing infectious material in areas of forests being threatened by the insect. This was accomplished in several different ways: by collecting forest litter from areas in which the disease had recently occurred and then transferring this material to uninfected areas; by gathering diseased and dead larvae, triturating them and sprinkling the resulting suspension on living larvae which were then freed; by feeding infectious material to larvae just before releasing them to mingle with the remainder of the population; by spraying the trees with infectious material; or by shooting such material into the crown of the trees by means of mortar guns adapted to this purpose. These methods brought only partial success in controlling the insect, but Růžička goes on to explain how these methods might be improved so as to give more promising results. The year following Růžička's report, Klöck (1925) described a successful artificially induced epizootic among nun-moth larvae in a Bavarian forest. He used the method of bringing in forest litter containing relatively large numbers of polyhedra.

Růžička (1925) considers climate to be the principal factor in determining an outbreak of *Wipfelkrankheit* in the nun-moth caterpillar. Dry hot springs favor the insect, while damp cold air encourages the disease.

The possibility of predicting the extent of an epizootic of *Wipfelkrankheit* in a given population of nun-moth larvae is mentioned by Bergold (1943). This author suggests that collected eggs could be hatched

during the winter and that since the hatched larvae would have acquired the virus from the eggs, the percentage of diseased larvae would be determined by microscopical examination. On the basis of such determinations a tentative prognosis as to the extent of the expected epizootic could be made.

The relations between the effect of insect parasites and that of *Wipfelkrankheit* on the natural control of the nun moth may be closer than is generally realized. In areas where tachinids parasitize the insect, usually it is the older larvae that are parasitized; but where the polyhedral disease also occurs, heavily diseased fifth-instar larvae are not parasitized (Niklas, 1939). The high incidence of virus disease in heavily infested areas causes many of the tachinids to migrate to the less infested areas. Gösswald (1934) observed that the insect parasite *Sarcophaga shützei* Kram. would not parasitize healthy larvae of *Lymantria monacha* Linn. and *Porthetria dispar* (Linn.) but did attack individuals suffering from polyhedral disease.

Since *Wipfelkrankheit*, unlike silkworm jaundice, is a disease of a destructive insect, no attempts have been made to suppress or control the disease. To the contrary, interested forest entomologists in Europe have done all that they could to promote the disease. Although the economic significance of the finding is not clear, it has been observed (Speyer, 1925) that the administration of arsenicals to nun-moth larvae appears to suppress the infection.

Polyhedrosis (Wilt Disease) of the Gypsy-moth Caterpillar

The gypsy moth, *Porthetria dispar* (Linn.), was brought to Medford, Massachusetts, in 1869 from the old world by a French naturalist who was concerned with the production of silk by different species of caterpillars. It soon extended its range until it occurred in most of the New England states where, to a large extent, the heaviest American infestations are still confined. The originally infested state of Massachusetts has in the past spent over a million dollars annually in efforts to control this serious pest of trees, both evergreen and deciduous. Prior to 1900 no evidence of any kind of infectious disease was observed in the American infestations, although diseased gypsy-moth caterpillars had been seen in Europe. Beginning about 1907, the disease under consideration began to be noticed in ever-increasing amounts. The question then arose: from where did it come? Glaser (1915) suggested several possible answers to this query, among which was the possibility that the disease may have been introduced from its original source in 1905, when the state of Massachusetts, in cooperation with the Federal Bureau of Entomology, imported large numbers of parasites and natural enemies of the gypsy moth from

Europe and Japan. The exact source of the disease probably can never be determined with certainty.

In the summer of 1907, Fiske (see Howard and Fiske, 1912) observed the wholesale destruction of half-grown caterpillars in several localities in Massachusetts and New Hampshire. At first there was some doubt as to the infectiousness of the malady, and some observers (Kirkland) believed that the disease was merely a natural condition resulting from overpopulation and that an insufficient or unsuitable food supply was the true cause. Evidence attesting to the transmissibility of the disease gradually accumulated, however, and it was soon looked upon as a possible means of controlling the insect. Howard and Fiske were of the opinion that the disease does not prevent the moth from increasing to an extent that renders it a pest but that it may, and frequently does, render very efficient service in effecting an enormous reduction in the abundance of the insect when other agencies fail. At the time of these early observations the disease in question was being popularly called the "wilt disease" or simply "wilt," and this name was taken up and used by subsequent authors.

Prior to Howard and Fiske's report, Reiff (1909*a*,*b*), attracted by the European accounts of an important disease in the nun moth (*Lymantria monacha* Linn.) and by Fischer's (1906) observations on the susceptibility of caterpillars to diseases, concerned himself with the experimental production of "flacherie" (believed to be bacterial in nature) in gypsy-moth caterpillars. In 1911, Reiff reported favorable results on extensive field tests in which he fostered the "flacherie" among gypsy-moth populations in several localities in Massachusetts. He used the terms "wilt disease" and "flacherie" synonymously; thus apparently he did not differentiate between the true wilt diseases, or polyhedroses, and the bacteria-caused "flacheries." He did, however, refer to natural epizootics of the disease in which case he was probably at the time dealing with the true polyhedrosis. Furthermore, from a scientific standpoint, so much of Reiff's work on this subject is open to criticism (see Escherich, 1913) that it is difficult to evaluate the effect, if any, that his experiments had on the clarification of the nature of the wilt disease of the gypsy-moth caterpillar.

It was not long after this before the wilt disease was generally recognized by foresters and entomologists alike as being a widespread infection in gypsy-moth larvae throughout the entire gypsy-moth-infested area in New England. The nature of the disease and its primary cause then became the subject of investigation by several men, chief of whom were Glaser and Chapman (1912–1916). The presence of the disease has also been recognized in western Europe and in Russia, where large numbers of the caterpillars are destroyed by it.

Symptoms. Wilt disease in gypsy-moth caterpillars manifests itself in a definite and distinct manner. The epizootic usually runs a very rapid course once it is underway, and 30 to 70 per cent of the insect population may be destroyed in any one outbreak. Dead caterpillars are usually found hanging anywhere on the trunk or on a branch of the tree. The sick insects never descend their food plants but usually search for an elevated place. They lose their appetite, become sluggish, and just before death they become soft and the internal tissues liquefy so that merely touching a dead insect ruptures the integument, and a dark-brown liquid oozes out. The caterpillars remain practically odorless until adventitious bacteria gain a foothold and the insect undergoes putrefaction. The period from infection to death varies from 4 to 24 days, with an average of 10 or 12 days.

Fig. 139. Caterpillars of the gypsy moth, *Porthetria dispar* (L.), dead of "wilt disease" and hanging from the bark of the host tree. (*Courtesy of R. W. Glaser.*)

Usually it is the older larvae that are visibly affected by the disease, but it has been reported from Europe that the disease kills more of the early instars than is generally assumed. Under certain conditions it appears that the disease may exist in a more or less chronic state. The chronic disease may become acute by the advent of environmental conditions that weaken or are disadvantageous to the host. Larvae infected in the last instar may survive to the pupal and even to the adult stage.

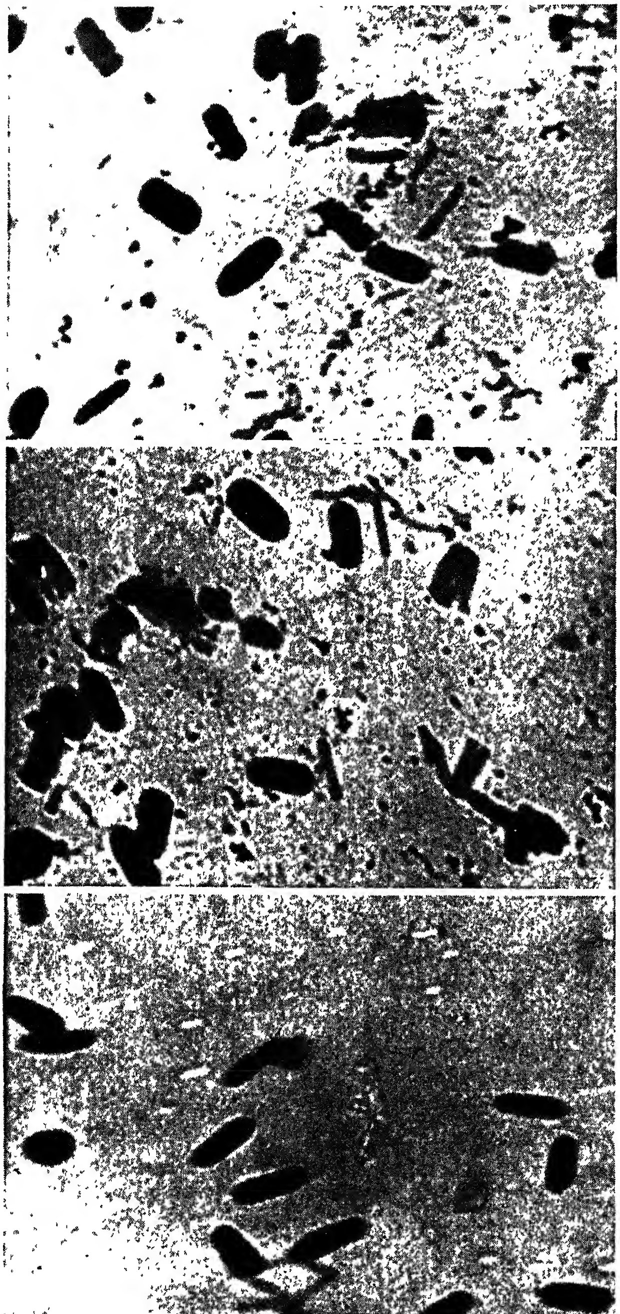
The Causative Agent. When it was suspected that wilt disease was infectious in nature, early investigators began to search for the causative microorganism. At first protozoa were looked for—which may have been prompted by the early, supposed protozoan etiology of silkworm jaundice and of *Wipfelkrankheit*. In making his examinations of diseased gypsy-moth larvae, Jones (1910) observed polyhedral bodies in the tissues and body fluids of his specimens, and Glaser and Chapman (1912) noticed them clustered around the tracheae of the insects. These bodies were observed to have a very high refractive index, to resist most stains, and to lack definite internal structure. It was concluded that the polyhedra

did not represent living microorganisms such as microsporidia, and Glaser and Chapman considered them to be reaction products of some sort. The latter investigators did, however, observe a small gyrating micrococcus in the tissues and the body fluids of the diseased caterpillars. They decided that this bacterium, which they named *Gyrococcus flaccidifex*, was the primary cause of the disease. About a year later, Glaser and Chapman (1913) published a reinterpretation of their observations and explained that the micrococcus was not the agent of the disease but was in fact "an intestinal invader pure and simple."

In their 1913 paper, Glaser and Chapman showed that the true cause of the wilt disease in gypsy-moth caterpillars was a filterable virus. They pointed out that bacteriologically sterile filtrates of the diseased larvae contained minute dancing granules that might have some connection with the virus, but they saw no reason for believing that the polyhedral bodies are stages of the filterable agent. Thus was established the fact that the primary cause of wilt disease was a filterable virus analogous to that causing jaundice in the silkworm.

Characteristics and Properties of the Virus and the Polyhedron. The virus of gypsy-moth wilt has not received so much detailed biochemical and serological study as has the virus of silkworm jaundice. It is probable, however, that the two viruses are similar in their essential characteristics. The gross properties of the virus of the wilt disease were listed by Glaser in 1918, and in recent years additional biochemical facts have been ascertained. It has been given the name *Borrelina reprimens* by Holmes (1948).

The virus is filterable through a Berkefeld N filter candle but does not pass a Chamberland F filter. If the filtrate is examined with a dark-field microscope, minute granules may be seen. These might be similar to those which occur in silkworm jaundice and may have etiological significance. The virus has not been cultivated on artificial media. Suspensions of the virus cause no fermentation of sugar solutions, no reduction of methylene blue or sodium nitrate solution, and no liquefaction of gelatin or of casein. It is destroyed in 20 minutes when held in moist heat at 60°C. Dry heat inactivates it at temperatures of 70 to 80°C. for 20 minutes. It remains viable for 2 years when held at room temperature and resists 98 per cent glycerin for 6 months. When the virus is dry, it resists the action of sunlight for 12 hours. The process of putrefaction does not appear to affect the activity of the virus. It is, however, destroyed by 80 per cent alcohol in 15 minutes, although it resists 5 per cent phenol for 3 weeks. A mixture of equal parts of 1 : 1,000 corrosive sublimate and 95 per cent alcohol, applied for 10 minutes, has been recommended by Glaser (1927) as being very effective in destroying the activity

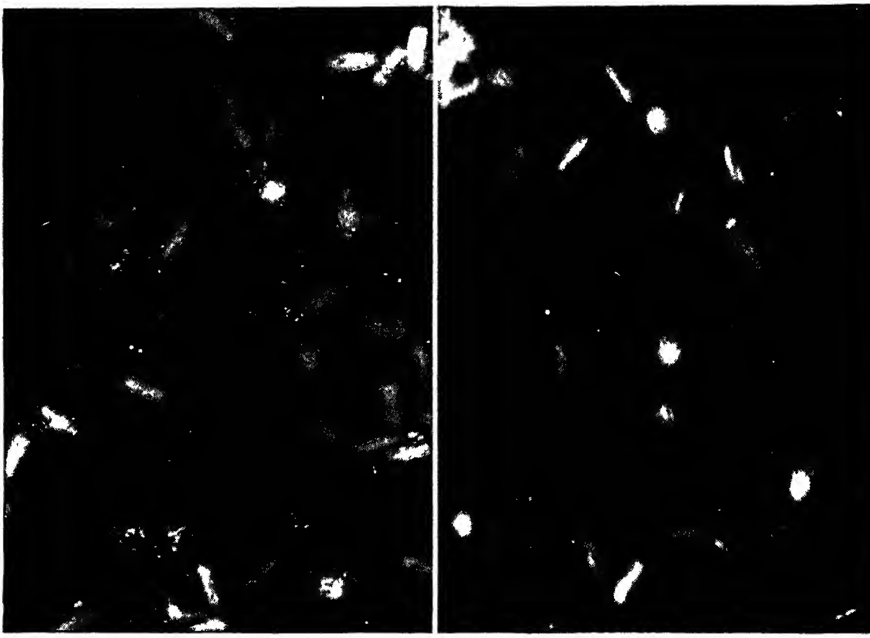


A B C

Fig. 140. Electron photomicrographs of the virus of gypsy-moth polyhedrosis taken at a magnification of about 30,000×. *A*. Suspension of virus bundles in water. *B*. Virus particles and bundles suspended in water. Some virus bundles are intact, others are becoming transparent, and still others are breaking down into the individual virus particles. Some of the virus particles occur singly. *C*. Another view showing virus bundles as well as single elements. (*Courtesy of G. Bergold.*)

of the virus. It is also inactivated by polyvinylpyrrolidon ("Kollidon") (Bergold, 1948*c*).

As shown by Bergold (1947, 1948*b*), the wilt virus is a rod-shaped particle having an average size of approximately 41 by 360 millimicrons. As seen with the electron microscope, one very interesting characteristic of the wilt virus, as of certain other insect viruses, is that it appears to



D *E*

Fig. 140 (*Continued*). *D* and *E*. Preparations of the virus of gypsy-moth polyhedrosis showing separating bundles and individual particles. (*Courtesy of G. Bergold.*)

consist of several rod-shaped particles lying together like a bundle of cigars. At certain points along each of the rod-shaped particles making up a bundle, spherical portions, or nodes, directly opposite each other can sometimes be seen when two or more particles are still hanging together. This may suggest some sort of sideways or longitudinal multiplication. Other characteristics of the virus include its having a Svedberg sedimentation constant s_{20} of between 2,500 and 4,000 (depending on the number of particles adhering together), a diffusion constant of 0.175×10^{-7}, a frictional ratio (f/f_0) of 1.42, an axial ratio of 8.8, a particle weight of 1300×10^6 when calculated from the sedimentation and diffusion constants, and of 391.6×10^6 when calculated from the length and diameter of the virus particle as seen in electron micrographs. Chemically speaking, the

virus is essentially a nucleoprotein of the desoxyribonucleic acid type. It is infectious to 10^{-10} gram of protein per larva.

The average polyhedron of gypsy-moth wilt disease measures about 3.5 microns in diameter, although variations from 0.5 to 15.0 microns have been reported. They are crystallike in appearance but are not so regularly hexagonal as the polyhedra of silkworm jaundice, nor are all corners so angular as are those of the latter. As with the polyhedra of jaundice, those of gypsy-moth wilt disease will crack on pressure, and they appear to consist of concentric onionlike layers. They may frequently be seen adhering to one another as if in the act of dividing. They are insoluble in hot or cold water, ether, chloroform, alcohol, or xylol. They dissolve in acids and weak alkalies. Picric acid stains them yellow, indicating their protein nature; further chemical tests have shown them to be of the nature of nucleoproteins. They do not stain with Sudan III or blacken with osmic acid, indicating that they contain no fat. They do contain iron and phosphorus. According to Bergold (1948*b*) the principal component of the polyhedral protein has a Svedberg sedimentation constant s_{20} of 12.57 and a molecular weight of 276,000. The split components have a sedimentation constant of 3.12 and a molecular weight of 47,250.

Fig. 141. Polyhedral bodies characteristic of the polyhedrosis of the gypsy-moth caterpillar. Photomicrograph of a smear preparation. (*From Glaser, 1915.*)

In their observations on the biochemistry of the polyhedra, Glaser and Chapman (1916*a,b*) studied the solubility of these bodies in various reagents. They found that 37 per cent hydrochloric acid, used both hot and cold, dissolves the polyhedral bodies with difficulty. The bodies dissolve in boiling nitric acid in solutions of between 15 and 31 per cent—14 per cent does not affect them and in 31 per cent they dissolve instantly. Ammonium hydroxide does not appear to affect the polyhedra, but such alkalies as potassium hydroxide and sodium hydroxide dissolve them readily. If boiled in a solution of sodium hydroxide as low as $\frac{1}{16}$ per cent, they will dissolve. A 2 per cent solution is a convenient concentration for making solubility tests. Glaser and Chapman found that, on dissolving the polyhedra in alkali and after dialyzing away the alkali and evaporating the protein solution, crystals are obtained which simulate

the original polyhedra. It is interesting that, when the polyhedra are treated with sodium carbonate, they may be found to have swollen to double their normal size, and when held in certain concentrations (*e.g.*, $0.008M$) of this chemical they dissolve.

The polyhedra are rather resistant to most stains and usually color around the periphery only. If stains are applied for prolonged periods of time or along with heat or a mordant, the polyhedra take the dyes rather well and usually uniformly. Sometimes, however, the stain reveals

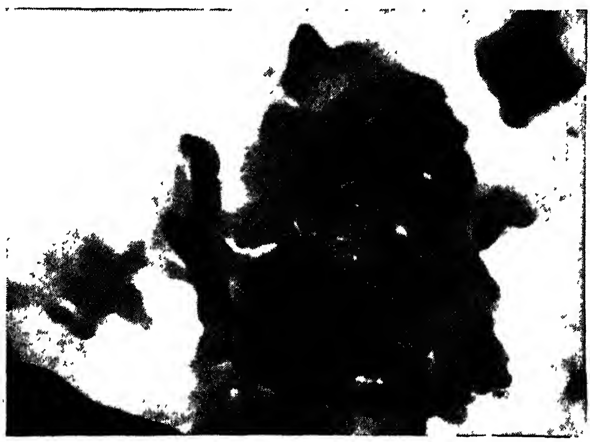


Fig. 142. Partly dissolved polyhedral body from diseased gypsy-moth caterpillar, showing cavities left by escaped virus particles or bundles. Polyhedron dissolved in dilute sodium carbonate solution. Electron photomicrograph taken at a magnification of 25,000×. (*Courtesy of G. Bergold.*)

tiny refractive granules within the polyhedra, and sometimes the polyhedron appears with a uniformly darker staining, central mass which can be differentiated from an almost unstained outer substance (Glaser and Chapman, 1916*a*,*b*).

Pathology. The gross pathology of wilt disease in gypsy-moth caterpillars consists principally of the "wilted" or flaccid appearance of the entire caterpillar, which is usually found hanging on the branches or trunk of the tree. If disturbed, the integument ruptures, permitting the dark-brown fluid contents to flow out. At this time the dead insect has no pronounced odor, although later, after bacterial decomposition has set in, it may have an unpleasant odor. Before the invasion of adventitious bacteria the contents of the diseased caterpillars are frequently free of bacteria. The internal tissues are practically all disintegrated, although the intestinal tract is one of the last of the internal organs to break down. If a diseased caterpillar is dissected and examined before death, one is likely to notice that the tracheae and their finer branches have grapelike

clusters of rounded bodies attached to them. These clusters consist of masses of polyhedral bodies within the nuclei of the tracheal matrix cells—one of the first tissues to be affected. Not many other gross pathological changes are to be noted.

As was explained in the case of silkworm jaundice, the polyhedra arise in the nuclei of the hypodermal, fat, tracheal matrix, and certain blood cells, and most of the pathology is concerned with these tissues. Breindl (1938) maintains that nerve and muscle tissue is also infected. The histopathology of gypsy-moth wilt is essentially the same as that which we have described for the silkworm disease. A fairly complete account of the pathological changes in the various cells and tissues has been given by Glaser (1915).

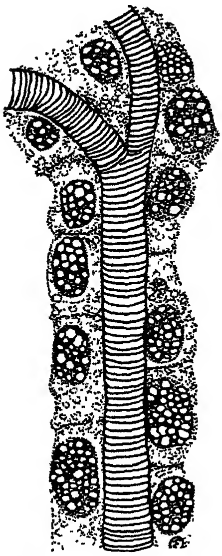


Fig. 143. Part of a tracheal tube of a diseased gypsy-moth caterpillar showing the presence of polyhedra in the nuclei of the cells of the tracheal matrix. (*Adapted from Glaser, 1915.*)

The first discernible change in the nucleus of an affected cell is the flowing together of the chromatin into a clump in the middle and the appearance of numerous dancing granules within it. These granules stain a reddish color with Giemsa's solution. It is sometimes difficult to distinguish these from the granules that appear when normal cells are permitted to degenerate. Perhaps in light of Paillot's work on the granules of silkworm jaundice (see page 429), those of wilt disease need reinterpretation. Out of the achromatic substance of the nucleus the polyhedral bodies may be seen to form—at first they are extremely minute in size, but they gradually increase in both size and number. The nucleus hypertrophies to an enormous size until finally the nuclear membrane breaks and the cytoplasm is destroyed, permitting the polyhedra to escape into the body cavity where they are found free in great numbers.

Although polyhedra do not form within the nuclei of muscle, nerve, excretory, and glandular cells, Glaser observed that some changes do occur. The chromatin of these cells, for example, shows signs of degeneration, and it may flow together into clumps. The minute reddish-staining granules, however, are not found within these cells.

The blood cells, or hemocytes, of infected caterpillars also undergo pathological changes—especially in the leucocytes and lymphocytes. (Polyhedra are not found in the spherule cells or in the oenocytoids.) The changes noted are essentially the same as those seen in the nuclei of

other tissue cells. The dancing granules appear as well as the polyhedra, which eventually break out of the hypertrophied nucleus and occur freely in the hemolymph. Occasionally cells appear with only one or a few polyhedra in them, in which case they may have been phagocytosed. Glaser (1915) described one type of pathological blood cell in which the entire cytoplasm of the cell seems to have disappeared and all that remains is the cellular membrane and a nucleus containing several polyhedra. Glaser suggests that the cytoplasmic material might have been used as

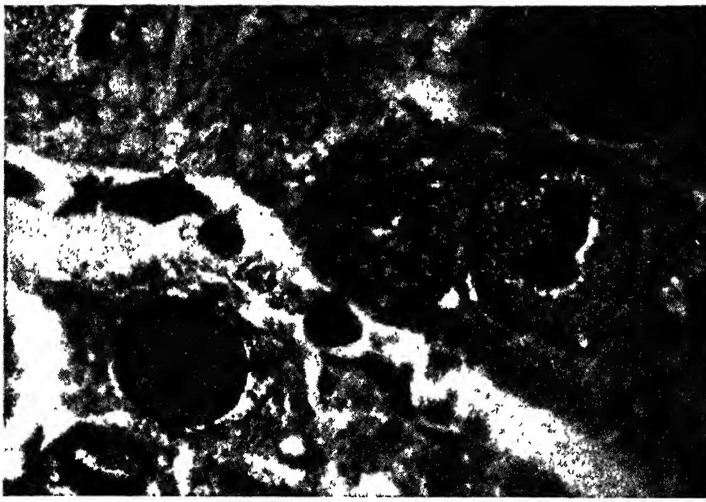


Fig. 144. Photomicrograph showing various stages during the formation of the polyhedra of gypsy-moth wilt in the tissue nuclei of a gypsy-moth caterpillar. (*From Glaser, 1915.*)

nutriment by the virus, but such would be an unusual type of nutritional arrangement. Because of the changes observable in the hemocytes at the earliest stages of the disease, the use of the blood as an indication of infection is a fairly reliable test for diagnosis.

Transmission. Transmission ordinarily occurs by way of the insect's alimentary tract, possibly passing through the intestinal epithelium into the hemolymph. Transmission from generation to generation may take place via the egg in a small proportion of individuals (Glaser, 1927).

Since the food plants of the gypsy moth are easily contaminated by the disintegrating bodies of the diseased insects, it is obvious that the virus is distributed in a manner making it readily acquired by healthy insects along with their food.

It has been suggested that insect scavengers may play an important role in the dissemination of the virus. Allen (1916) points out that Sarcophagidae are particularly attracted to the disintegrating larvae and pupae

and breed freely in them. Polyhedra have been found on the legs and mouthparts of these insects. Other insects have also been observed to frequent the site of the diseased caterpillars. Elaterids, coccinellids, certain Hemiptera and Coleoptera including *Calosoma sycophanta* Linn. larvae, and even ants and mites, have been found in this association and observed to carry polyhedra mechanically on their bodies.

The virus apparently does not depend upon the wind for its distribution.

Immunity. It appears that a certain number of caterpillars in any one population are not susceptible to the disease. Whether this is actually an acquired humoral immunity, an apparent immunity based on physiological characteristics of individual caterpillars, or merely coincidental has not been determined. In any case, it is an interesting means of protection possessed by the insect species for surviving widespread epizootics. It may be one of the reasons why complete eradication of a species by a virus disease is highly improbable, even though adequate control of the pest is feasible by this means.

Glaser (1915) reports that out of 195 gypsy-moth caterpillars fed with virus material, 57 adults emerged. He suggests that it is possible that a genetic immunity toward wilt exists among certain members of the gypsy-moth race and that others can also be actively immunized with sublethal doses of fully virulent material. Glaser tells of field observations in which he saw large numbers of caterpillars congregating on trees under burlap and many of them dying of wilt in such places. Yet, in spite of the disintegrating bodies flowing out over other individuals in the immediate proximity, many will escape death and transform into adults.

In general the virus of gypsy-moth wilt appears to be distinct from other insect viruses and fairly well limited to its specific host. Experimentation has shown such insects as the tent caterpillar (*Malacosoma americana* (Fabr.)), and the silkworm (*Bombyx mori* (Linn.)) to be refractory. On the other hand, Bergold (1943) reports that the silkworm is at least partly susceptible to the gypsy-moth wilt virus and that in his tests some silkworms died after oral infection with a polyhedral suspension and after intralymphal infection with dissolved polyhedra. Larvae of *Lymantria monacha* Linn. and *Dendrolimus pini* Linn. also appeared to be somewhat susceptible. The polyhedra found in the test animal were usually characteristic of the host insect. In some cases gypsy-moth larvae were infected with the virus of *Dendrolimus pini*; typical *D. pini* polyhedra were found. Gypsy-moth larvae also appeared to be susceptible to the viruses of *B. mori* and *L. monacha*. Further experimentation along this line is needed, however, before definite conclusions can be drawn as to the interspecies susceptibility to polyhedral viruses.

Polyhedroses in Other Lepidoptera

The number of species of Lepidoptera known to be susceptible to infection by the polyhedral viruses is already a large one—approximately 100. Naturally this number is undoubtedly only a small fraction of those which actually exist. With more accurate observing and better reporting the number will probably increase rapidly. For the present, however, it is still convenient to mention in discussion form most of the insect species concerned. Unfortunately, all cases of so-called "polyhedral disease" or "wilt disease" have not been substantiated by microscopic demonstration of polyhedral bodies. In such instances we can but record what the literature contains and wait until such observations are confirmed.

On the following pages, the various species of Lepidoptera that have been reported as being susceptible to polyhedroses are arranged systematically according to families. This arrangement will give the reader some idea as to the relative number and extent of the observations made in the various groups. He should remember of course that detailed discussions have already been presented concerning the polyhedroses of the silkworm, the nun-moth caterpillar, and the gypsy-moth caterpillar. So as to enable the research worker to find the original or more complete reports, the following discussions are necessarily encumbered with the citation of many of the source references. This is not to imply, however, that the treatment is bibliographic in the complete sense. An attempt has been made to cite the principal or most pertinent references in each case.

Tineidae. The larva of the webbing clothes moth, *Tineola bisselliella* (Hum.), is occasionally found attacked by a polyhedral virus, particularly when reared in the laboratory.

In Basel, Switzerland, Lotmar (1941*b*) observed larvae of the clothes moth to be infected with a polyhedrosis in which the polyhedra were similar in appearance to those seen in silkworm jaundice. Larvae could easily be infected by allowing them to feed on wool contaminated with crushed infected larvae. The diseased larvae usually died, but one specimen matured to a female adult, which gave rise to progeny that were not infected. Lotmar (1941*a*) also noticed a microsporidian (*Nosema*) infection in the larvae of this insect.

Oecophoridae. In 1945, Harrison reported the occurrence of a polyhedral disease in larvae of *Chimabache fagella* Fabr. in England. The specimens that had become diseased were collected from one particular locality in Lamesley, County Durham; specimens collected from other areas remained disease-free when brought into the laboratory. The polyhedra were demonstrated microscopically.

Phaloniidae. According to Chapman and Glaser (1915), *Phalonia ambiguella* (Hbn.) has been cited by European workers as being susceptible to a polyhedrosis.

Tortricidae. The black-headed budworm, *Acleris variana* (Fern.), is attacked by a polyhedrosis in British Columbia, the state of Washington, and probably elsewhere. In some areas a considerable percentage of the larvae is destroyed by the disease.

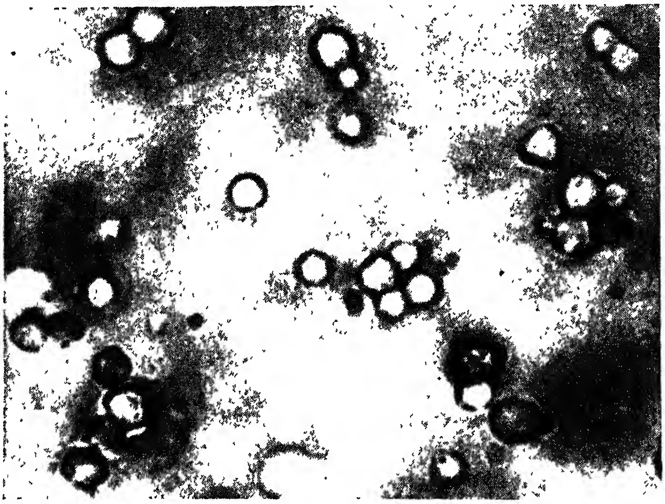


Fig. 145. Polyhedral bodies characteristic of the polyhedrosis in the black-headed budworm, *Acleris variana* (Fern.). (*Photograph by K. M. Hughes and J. M. Smith.*)

A polyhedrosis of the spruce budworm, *Choristoneura fumiferana* (Clem.), has been observed by Graham (1948) who records the interesting fact that the polyhedra originate in the "digestive cells of the midgut." In addition, there is an excessive, malignant multiplication of the midgut cells, accompanied by a contraction of the larva's muscles, which causes the insect to shrink in length.

The tea tortrix, *Homona coffearia* Nietn., has been reported (Stockdale, 1920) as subject to outbreaks of a polyhedrosis in Ceylon when the insect becomes overcrowded. Under such conditions large numbers of the pest are destroyed. Attempts to produce outbreaks of the disease artificially have not succeeded.

The larch tortrix, *Enarmonia diniana* Gn., in Italy apparently suffers from a polyhedral infection, according to Del Guercio (1929).

It might be mentioned here that the codling moth, *Carpocapsa pomonella* (Linn.) (family Olethreutidae), was observed in 1920 in Delaware by Selkregg and Siegler (1928) to be killed by "an unidentified wilt disease." This could have been bacterial rather than virus in origin. In

southern France, however, the cocooned larvae of this insect were definitely reported to be attacked by a polyhedral disease (Simmonds, 1944).

Limacodidae. In 1931, King reported that in Ceylon nettle grubs (*Narosa conspersa* Wlk., *Natada nararia* Moore, *Thosea cervina* Moore, *T. recta* Hmps., *T. cana* Wlk., *Parasa lepida* Cram., and *Spatulifimbria castaneiceps* Hmps.) readily succumb to wilt diseases at certain seasons of the year. Experiments were conducted to propagate the diseases artificially by spraying larvae with water in which infected caterpillars had been macerated. The results were described as being very encouraging. In 1933 King reported that "wilt" disease appeared to kill larger numbers of the pests than all other natural enemies combined.

Smee (1940), in Nyasaland, described a "wilt" disease of the gelatin grub of tea, *Niphadolepis alianta* Karsch, which killed the majority (88 per cent) of active larvae in June and July of 1939. Over the entire year only about 12 per cent of the larvae were killed by the disease, but 1940 records up to the end of April showed a mortality of 48.9 per cent. The pupae may also be diseased.

Unfortunately no recording of polyhedral bodies in any of the insects just named has been made, but from other characteristics of the diseases it is assumed that polyhedral viruses were involved.

Arctiidae. In 1915, Chapman and Glaser included the fall webworm, *Hyphantria cunea* (Drury), in a list of insects having diseases similar to "wilt" in many of their clinical aspects. *Callaractia virgo* Linn. may also be susceptible to a polyhedrosis. According to G. R. Wyatt, the larva of the great tiger moth, *Arctia caja* (Linn.) very definitely is subject to such a disease. Polyhedra have been observed in the salt-marsh caterpillar, *Estigmene acraea* (Drury), in California.

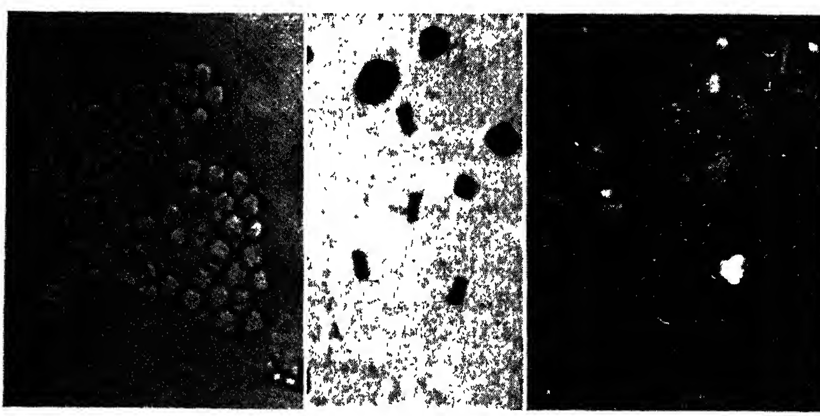
Psychidae. In South Africa, the wattle bagworm, *Acanthopsyche junodi* Hely., is affected by a polyhedral wilt disease as well as by fungous infections (Skaife, 1921). Climatic conditions greatly influence the activity of these diseases.

Noctuidae.[1] The western yellow-striped armyworm, *Prodenia praefica* Grote, occurs throughout central and northern California, western Nevada, and southern Oregon, principally on alfalfa. On frequent occasions, a polyhedrosis breaks out among the insects and may destroy large numbers of them. In fact, Blanchard and Conger (1932) state that the polyhedral disease was the most important factor in natural control as observed by them (see also Cartwright and associates, 1933). As in most polyhedroses, the disease affects the larvae particularly in the fourth to last instars. The larvae may turn reddish-brown before death, after which the body contents disintegrate into a dark watery mass within the integument. The flaccid disintegrating insects may be found in large numbers hanging

[1] Recently changed to Phalaenidae.

from the leaves and stems of the host plants. The virus is a rod-shaped particle approximately 50 by 290 millimicrons in size; it apparently

Fig. 146. Two views of the armyworm *Prodenia praefica* Grote dead of polyhedrosis. (*Photographs by K. M. Hughes.*)



A B C

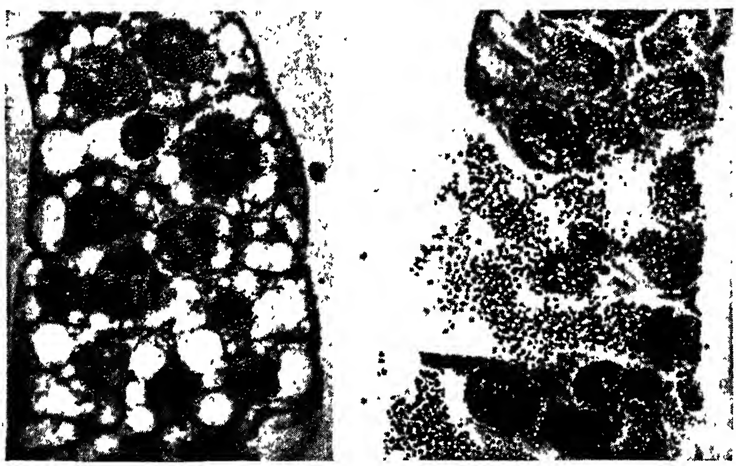
Fig. 147. Polyhedral bodies and virus characteristic of the polyhedrosis in the armyworm, *Prodenia praefica* Grote. *A*. A group of polyhedra. *B*. Characteristic virus bundles (and salt crystals). *C*. Individual virus particles gold-shadowed. Magnification of *B* and *C* approximately 12,000×. (*Photographs by K. M. Hughes.*)

occurs characteristically in small bundles of several members each (Fig. 147).

Another armyworm, *Prodenia ornithogalli* Guen., also is subject to attack by a polyhedral virus, as is *Prodenia litura* (Fabr.). The disease

was reported in the latter insect as early as 1913 in Egypt, where there was a heavy mortality of the insect as a result of the infection (Dudgeon, 1913). This insect has been similarly attacked in Indo-China where Caresche (1937) transmitted the disease to healthy larvae by feeding them on leaves treated with an extract from diseased individuals. The larvae treated in this manner died in 5 or 6 days. According to Crumb (1929), a disease, probably polyhedral, occurs in "*Prodenia litosia*" in Europe.

The fall armyworm, *Laphygma frugiperda* (A. & S.), was listed by Chapman and Glaser (1915) as being susceptible to a polyhedral disease.



A *B*

Fig. 148. Histological sections of tissues from the cosmopolitan armyworm, *Leucania unipuncta* (Haw.), suffering from polyhedral infection. *A*. Polyhedral bodies within the nuclei of the fat cells. *B*. A section of hypodermis showing the breakdown of infected tissue and the liberation of the polyhedra. (*Courtesy of R. W. Glaser.*)

In 1921 Allen recorded an outbreak of polyhedrosis in this insect in Mississippi where it was very abundant during the summer of 1920. The disease was first noted in September among larvae in the last instars. Dead larvae were found hanging from the tips of blades of grass in considerable numbers. The freshly dead insects were yellowish in color, and their internal tissues had become completely disorganized and liquefied. The presence of irregularly angular polyhedra was confirmed by microscopic examination. Collected but separated specimens brought into the laboratory showed a mortality of 37 per cent.

The cosmopolitan armyworm, *Leucania unipuncta* (Haw.) (*Cirphis unipuncta* (Haw.)), has been found diseased in Massachusetts, Maryland, Virginia, North Carolina, Illinois, Oklahoma, and California. A virus disease has been credited with keeping the insect controlled in Rhodesia.

In the New England states, Chapman and Glaser (1915) observed that the larvae were flaccid, and upon death they hung by their prolegs, their integument being so fragile that it broke at the slightest touch, releasing a thin grayish fluid filled with polyhedral bodies. Diseased specimens that have been examined in California have shown polyhedra with an average size range of 1.2 to 3.5 microns and with an irregular number of sides, usually 4 to 6.

Heliothis armigera (Hbn.), a pest of corn, cotton, and tomatoes, was listed by Chapman and Glaser (1915) as subject to wilt disease. As early as 1891, Mally referred to diseases of this insect as well as to those of *Prodenia ornithogalli* Guen., *Euxoa messoria* (Harr.), *Nephelodes emmedonia* (Cram.), and others. It is possible that some of these instances were actually polyhedroses, but since the presence of polyhedral bodies was not generally recognized in such insects at that time, no factual data exist to clear up this point of Mally's report. Several strains of bacteria were isolated, however, and the cause of the infections was attributed to these microorganisms. (That a polyhedrosis does occur in *Nephelodes emmedonia* (Cram.), the bronzed cutworm, is indicated by such accounts as that of Walkden (1937), in which this author states that 32 per cent of the larvae under his observation died of a wilt disease. A definite diagnosis of "polyhedral disease" in the bronzed cutworm has been made in Ohio according to a personal communication from J. S. Houser.) Something other than a typical polyhedral virus was apparently involved in some cases, since adults as well as larvae and pupae of *Heliothis armigera* (Hbn.) were sometimes observed to suffer from an infection in which the adult moths ordinarily resistant to virus infection became sluggish in movement and acquired greatly distended abdomens. The abdomens became decomposed, and the last signs of life were "peculiar alternate openings and closings, contracting and expanding of the anus and genital organs."

In the laboratory, Stahler (1939) found 10 to 100 per cent mortality to occur in larvae of *H. armigera* (Hbn.) reared in cages. The diseased caterpillars assumed a metallic luster, stopped feeding, became generally paralyzed, and failed to molt. Upon death, the cuticle blackened and became soft and sticky so that the least tension pulled it apart, allowing the liquefied internal contents to flow out. Most of the diseased specimens were in the later instars, although a few were seen infected in the second instar. When the larvae were fed on lettuce or on alfalfa the mortality was higher than when they were fed on tomato fruits or on corn meal. Stahler believed he was concerned with a "wilt disease," but he saw no polyhedral bodies. There is reason to believe, however, that these may have been overlooked.

Heliothis "obtectus" is also apparently subject to attack by a "wilt disease" (Lounsbury 1913*a*), and polyhedra have been observed in *Heliothis phloxiphaga* Grt. & Rob. in California.

In 1912, Hyslop mentioned the occurrence of a disease in larvae of the alfalfa looper, *Autographa californica* (Speyer), in the state of Washington. The infected insects were described as turning black and becoming a limp mass at the time of death. This disease he observed is now considered to have been a polyhedrosis. It also occurs in California and in British Columbia. In the case of *Trichoplusia ni* (Hbn.) (*Autographa brassicae* Riley), polyhedra were first demonstrated in 1915 (see Chapman and Glaser, 1915). The disease caused by this virus has also been detected in Russia. Polyhedral bodies have also been demonstrated in *Autographa biloba* Steph. on lettuce in Mississippi (Allen, 1924). The cotton leafworm, *Alabama argillaceae* (Hbn.), apparently also suffers from a polyhedrosis.

In Europe the cutworm *Euxoa segetum* (Schiff.), is attacked by a polyhedral virus that multiplies in the cells of the tracheal matrix, hypodermis, and fat tissue. Polyhedra have not been observed in the nuclei of the blood cells. The lesions first appear in the tracheal cells, and the virus appears to have the greatest affinity for these cells. The polyhedra are triangular in shape and are usually 3 to 5 microns in diameter. Virus particles or elementary bodies similar to those seen in silkworm jaundice have been observed in the blood of infected cutworms. What makes this polyhedrosis particularly interesting is the fact that there is practically no mortality from the disease and the morbidity is less than 1 per cent in regions where it is found. In fact, infected cutworms are very difficult to distinguish from healthy ones; the hemolymph is not very turbid and contains few polyhedra. Paillot (1936) discovered this disease in France in 1935. After examining hundreds of larvae he found only 10 to be infected. Apparently no epizootics of the disease occur. The infection is sometimes seen in conjunction with a nonpolyhedral disease of the same insect (see page 503). One is tempted here to speculate as to whether this polyhedral virus, if specific for its host, is of inherently low virulence or whether it is in the process of becoming commensally adapted to its host. Its low virulence might also conceivably be attributed to the possibility that *Euxoa segetum* (Schiff.) is not its specific host but that it is nevertheless susceptible to it to a small degree.

A disease of unknown etiology, possibly a polyhedrosis, is described by King and Atkinson (1928) for the red-backed cutworm, *Euxoa ochrogaster* (Guen.).

Strickland (1916) describes a disease of the army cutworm, *Chorizagrotis auxiliaris* (Grote), in which the larvae become inflated and turn

brown and the body contents become liquefied and decomposed. It is possible that this is a polyhedral infection. Another disease of this insect turns the larva red and is probably bacterial in origin.

Other noctuids that have been reported as being susceptible to polyhedral viruses include the well-marked cutworm, *Noctua clandestina* Harr., and the pine-moth larva, *Panolis flammea* Schiff. The latter species has been reported as subject to a polyhedral disease in Holland (Ritzema Bos, 1920), and in Poland (Sitowski, 1924). In New Zealand, according to Miller (1929), the larva of the cinnabar moth, *Tyria jacobaeae* Linn., is subject to attack by a polyhedral disease, especially when the insect is reared in the insectary.

Dioptidae. The California oakworm, *Phryganidia californica* Pack., is one of the most destructive of the defoliating pests of the live oak and the valley oak in California. This insect has long been known to suffer from a disease thought by some (*e.g.*, Burke and Herbert, 1920) to be bacterial in nature but now known to be a polyhedral virus disease. Chapman and Glaser (1915), and others have demonstrated the polyhedra in the tissues of the diseased insects. When the insect is present in large numbers, the disease may appear quite suddenly and kill off thousands. The symptoms of the infected insects are essentially similar to those of the other polyhedroses.

Notodontidae. The saddled prominent, *Heterocampa guttivitta* (Wlkr.), is a pest of forest trees in northeastern United States. In 1907 a serious outbreak of caterpillars occurred in Maine, and wherever the population was extremely dense, the insects were attacked by a "contagious disease" (Patch, 1908). Although at first thought to be caused by a fungus, it was later shown to be a polyhedral disease. Collins (1926) records the observation of polyhedral bodies in diseased material collected in Massachusetts in 1919. Large numbers of dead larvae were also seen in New Hampshire in the same year.

Another notodontid, *Cerura bifida* (Hbn.), has been recorded as susceptible to a polyhedrosis (Chapman and Glaser, 1915), and *Stauropus alternus* Wlk. might also be subject to such an infection.

Lymantriidae. The two members (*Lymantria monacha* Linn., and *Porthetria dispar* (Linn.)) of this family that have figured most prominently with respect to their polyhedral diseases have already been discussed in some detail (see pages 449 to 464). Several other species have also been observed to suffer from polyhedroses, but none of these has had as detailed a study as the two species just named. Chapman and Glaser (1915) report such observations with caterpillars of the white-marked tussock moth, *Hemerocampa leucostigma* (A. & S.). Although it was seen earlier by Howard and Fisk (1912), who also reported a "wilt" disease

in the "pine tussock moth," Chapman and Glaser observed the disease in 1911 in Massachusetts, where it almost completely wiped out the second generation of caterpillars in the area concerned. In 1914 they again recog-

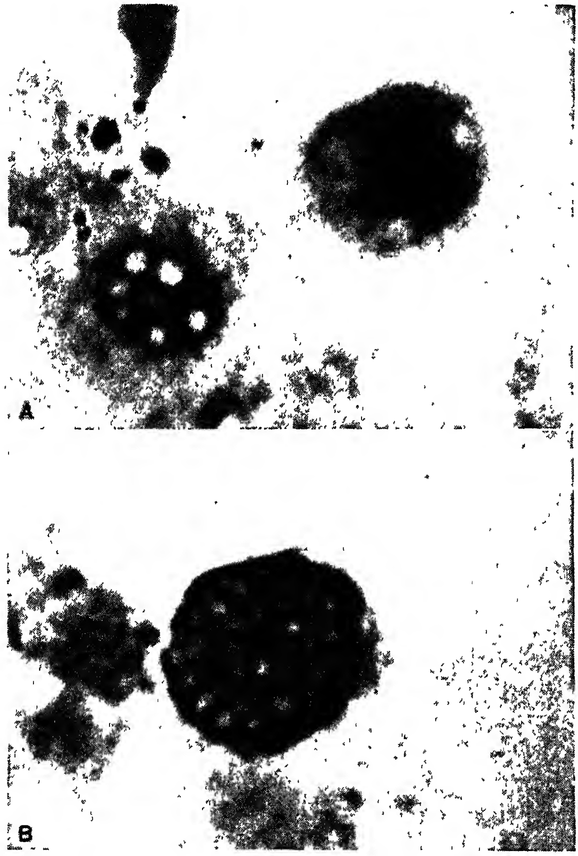


Fig. 149. Blood cells from larva of the tussock moth, *Notolophus antiqua* (Linn.), infected with polyhedral virus. *A*. The nucleus of one of the cells contains well-formed polyhedra. *B*. Later stage in which the polyhedra-filled nucleus is greatly hypertrophied. (*Photograph by K. M. Hughes and J. M. Smith.*)

nized the infection in caterpillars sent to them from Washington, D.C., and from Wooster, Ohio. In both cases they demonstrated the presence of polyhedral bodies. The Ohio outbreak occurred at a time when the caterpillars were supposed to be transforming to the pupal stage. Enormous numbers of the insects were destroyed. Infestations of Douglas-fir tussock moth, *Hemerocampa pseudotsugata* McD., have been greatly reduced because of a polyhedrosis affecting this insect. In forests in

northwestern United States mortalities of 60 to 75 per cent have been frequently observed, and in some areas almost the entire infestation has been wiped out.

Furniss (1939) refers to a "wilt" that killed full-grown satin-moth larvae, *Stilpnotia salicis* (Linn.), in Tacoma, Washington. The rusty tussock moth, *Notolophus antiqua* (Linn.) (and the subspecies *badia*), as well as the brown-tail moth, *Nygmia phaeorrhoea* (Donov.), have also been observed to be susceptible to virus infections. Outbreaks of the brown-tail moth have been brought under control in Europe by the natural occurrence of polyhedral disease (*e.g.*, see Zwölfer, 1925). According to Tooke (1938), the pine brown-tail moth, *Euproctis terminalis* Walk., on pines in eastern Transvaal, was attacked by a polyhedral disease in 1930. He believes that this insect is normally highly resistant to the disease.

In Germany, *Dasychira pudibunda* Linn. was reported attacked by a polyhedral disease in 1917 and 1918 (Krausse, 1919). The same disease has been reported from other parts of Europe (*e.g.*, Belgium), and similar reports have emanated from Finland (Linnaniemi and Hukkinen, 1921) with regard to *Dasychira selenitica* Esp.

Sphingidae. While studying several species of sphinx moths in Vienna, Böhm (1910) observed an outbreak of polyhedral diseases among them, which he described as being similar to the *Wipfelkrankheit* of the nun moth. The sphingids lost their appetite, became sluggish in movement, and finally hung to the walls or tops of their rearing cages and died. When disturbed, the body walls broke easily, freeing a disagreeably smelling fluid that contained large numbers of polyhedral bodies. The polyhedra were cubical in shape and in cross section appeared as a regular square. The species of sphingids with which Böhm worked are as follows: *Deilephila vespertilio* Esp., *D. galli* Rott., *D. euphorbiae* Linn., *Pergesa elpenor* Linn., *Proserpinus proserpina* Pall., and the hybrids *Deilephila phileuphorbia* Mütz., *D. kindervateri* Kysela, and *D. harmuthi*. Whether or not Böhm observed polyhedral infections in all these insects or only in certain ones is not clear from his report.

Smerinthus atlanticus auct. has also been reported as susceptible to a polyhedral infection (Chapman and Glaser, 1915).

Geometridae. The western hemlock looper, *Lambdina fiscellaria lugubrosa* (Hulst) (*Ellopia*), is known to be heavily diseased at times. Larvae collected in Oregon in 1945 were observed to be affected by a typical polyhedral disease. *Lambdina somniaria* (Hulst) has been found similarly infected (Wyatt, 1946), as has the false hemlock looper, *Nepytia canosaria* Walk., in British Columbia. In 1910 Wolff, in Europe, reported that *Bupalus piniarius* Linn. is subject to a polyhedral disease.

The geometrid *Ptychopoda serriata* Schrk., sometimes placed in the

Geometridae and sometimes in Acidaliidae, has also been reported to suffer from a polyhedrosis (Bergold, 1943). The polyhedra are irregular or triagonal in shape, and their average size is from 2 to 6 microns across.

Lasiocampidae. The American, or eastern, tent caterpillar, *Malacosoma americana* (Fabr.), occurs in eastern United States; and the forest tent caterpillar, *Malacosoma disstria* Hüb., occurs throughout most of the continent. Both species were reported to be subject to polyhedroses

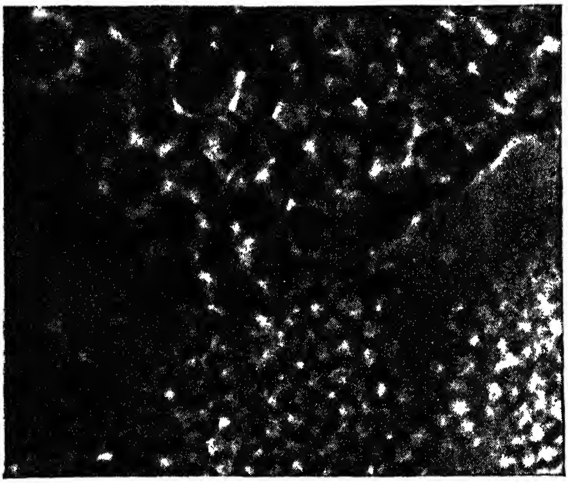


Fig. 150. Polyhedral bodies (two sizes) characteristic of the polyhedrosis of the western hemlock looper, *Lambdina fiscellaria lugubrosa* (Hulst). (*Photograph by K. M. Hughes and J. M. Smith.*)

by Glaser and Chapman (1913). One of the earliest reported outbreaks of the disease in tent caterpillars, however, is that seen by Fiske, who reported the occurrence of the disease in southern New Hampshire in 1898 (see Howard and Fiske, 1912). The outbreak seen by Chapman and Glaser in the American tent caterpillar was observed in 1914 near Lunenburg, Massachusetts, in trees in a low-growing swampy area. At first only a few caterpillars here and there were seen dying, and these were frequently spun over by the remainder of the colony. About the time that the trees had become completely defoliated, the disease broke out in epizootic form. Within the course of a few days, thousands of webs were covered with dead and dying caterpillars, most of which were strawberry-red in color. Polyhedra with more or less rounded angles were demonstrated in the brownish fluid of the diseased and disintegrating insects. The virus is filterable through Berkefeld V and N filters, passing the W filter only with difficulty, and the Pasteur-Chamberlain F filter

not at all (Glaser, 1927). When fed with the Berkefeld V and W filtrates, the larvae die in from 7 to 17 days. The virus is destroyed when submerged for 10 minutes in a mixture of equal parts of 1 : 1,000 corrosive sublimate and 95 per cent alcohol. There is evidence that in a small proportion of individuals the virus is transmitted from generation to generation with the egg. Tent caterpillars are not susceptible to the virus of silkworm jaundice, and the silkworm resists infection by the tent-caterpillar virus.

The disease is still being reported in the American tent caterpillar from the eastern part of the United States, where some states (*e.g.*, Connecticut) report up to 30 per cent mortality. In 1946 a wilt disease was effective in partly reducing the population of the forest tent caterpillar in the maritime provinces of Canada (Reeks, 1946). In all probability these diseases are likely to appear under the proper conditions wherever and whenever their hosts accumulate in sufficiently large numbers.

In 1943 Bergold described a polyhedrosis of the larva of the pine moth, *Dendrolimus pini* Linn. The size of the cube-shaped polyhedra associated with this infection was usually between 2 and 10 microns. Death resulted in from 13 to 28 days after oral infection. When last-instar larvae were given experimentally an infectious feeding, 17.6 per cent of them survived into the pupal or into the adult stage. There are strong indications that the virus can be transmitted to the next generation through the egg; at any rate the larvae of the next generation may acquire the virus in some manner from the egg. According to Bergold, *D. pini* larvae are partly susceptible to the viruses of the silkworm, the nun moth, and the gypsy moth. Also, except for the silkworm, these insects appear to be somewhat susceptible to the pine-moth virus.

Saturniidae. Those species of giant silkworms which have been found naturally infected with polyhedral viruses include *Saturnia pavonia major* Oliv. (reported by Conte and Levrat, 1909), *Hemileuca maia* Drury and *Hemileuca oliviae* Ckll. (reported by Chapman and Glaser, 1915), and the pandora-moth larva, *Coloradia pandora* Blake (reported by Wygant, 1941). In 1889, Bolle claimed to have experimentally infected the following insects with the virus of silkworm jaundice: *Antherea pernyi* Guer., *Antherea yamamai* Guer., *Antherea mylitta* Dru., and *Philosamia cynthia* Dru. Since most viruses are known to possess a marked degree of specificity for their specific hosts, there is good reason to doubt the accuracy of Bolle's results. Significantly, he found that in each insect the form of the polyhedra varied from that in the other insects. Hence it is possible that he was dealing with polyhedral diseases characteristic for each of the species inoculated and that the appearance of the disease in each case was more or less coincidental with the injection of the silkworm virus.

In South Africa, Tooke and Hubbard (1941) observed a polyhedrosis

of the pine-tree emperor moth, *Nudaurelia cytherea capensis* Stoll., which, together with a disease of unknown nature, caused a mortality approaching 90 per cent.

Pieridae. In the United States, *Colias philodice philodice* Godt., the clouded sulphur butterfly, occurs as a minor pest of clover in the eastern part of the country; in the west, and particularly in the southwestern United States, *Colias philodice eurytheme* Boisd., the alfalfa caterpillar, is one of the most important pests of alfalfa. Both of these insects are subject to attack by polyhedral disease. The first-named variety was listed by Chapman and Glaser in 1915 as being subject to "wilt." They further reported that the caterpillars did not appear to be susceptible to the virus of the armyworm, *Leucania unipuncta* Haw., when fed to the insects.

Fig. 151. Larva of the alfalfa butterfly, *Colias philodice eurytheme* Boisd., dead of polyhedrosis. Observe how fluid contents of body have gravitated to anterior end of insect. (*Photograph by K. M. Hughes.*)

The first reports of what was probably the polyhedral disease of *Colias philodice eurytheme* Boisd. are apparently those of Wildermuth (1911, 1914), who considered it the most common natural enemy of the caterpillar during 1910 in the Imperial Valley of California. He described the diseased caterpillars as assuming a lighter green color, becoming sluggish in movement, and hanging from the alfalfa stalks in soft, brownish-black, decaying masses. According to this worker, a first sign of the breaking down of the tissues may frequently be noticed when the larva is still active; this may consist of a slight exudation at some small broken place, usually toward the anterior end. In fact, the anterior end may be blackened and the posterior end still slightly moving, indicating that the insect is not entirely dead. Both pupae and larvae were affected but more often the larvae. The relatively strong pupal covering, however, usually prevents the "melting down" of the specimen; the decayed contents of the interior eventually dry up, leaving an empty but intact black shell. Wildermuth expressed the belief that the development

of the disease depends on moisture, since the malady occurred more often in moist fields than in dry ones. He surmised that one reason the caterpillar does not appear in such large numbers in certain regions of the southwestern part of the United States as in other sections is that the greater humidity of the regions provides the disease with greater opportunity to develop than is the case in drier areas. In his 1914 report he mentions that when conditions of moisture are produced artificially by irrigation, the disease is fostered to the extent of making it a factor in controlling the pest. Similar observations were made by Cartwright and associates (1933).

Wildermuth did not ascertain the true cause of the disease and assumed that it was bacterial in nature. Brown (1930) also attributed its cause to a bacterium (*Staphylococcus flaccidifex*), and Michelbacher and Smith (1943) considered it to be either a virus or a bacterium. (An enterococcus, *Streptococcus faecalis* And. & Hord., is a common inhabitant of the alimentary tract of the alfalfa caterpillar, and it is probably this microorganism which has caused some of the confusion in the past.)

One of the first reports that correctly ascribed the infection to a polyhedral virus was that of Dean and Smith (1935), who observed the disease in Kansas, the latter author having noticed it in 1927 and 1928. They state that the filterable virus invades the body of the caterpillar beginning with the third instar, although death of the larva may not occur until it is nearly grown. The infected caterpillar becomes yellowish in color or dotted with blackish spots. The body tissues disintegrate into a brownish fluid that is liberated when "finally the skin bursts." In agreement with other workers, Dean and Smith report that high humidity favors the development of the disease. The presence of polyhedra in similarly diseased caterpillars in California was noted in 1945 (Steinhaus, 1945). The virus is now known to be a small rod-shaped particle approximately 40 by 300 millimicrons in size as determined by electron micrographs (Fig. 152). As with certain other insect viruses, that of the alfalfa butterfly may occur singly or in bundles of several individuals each (Steinhaus, 1948). As observed in California, the first discernible symptoms of polyhedrosis in the alfalfa caterpillar usually begin to appear about 4 or 5 days after infection and consist of a lessening of the animal's appetite and of its general activity and mobility. Soon thereafter the normally green color of the larva changes to a pale yellowish or grayish green, sometimes giving the caterpillar a mottled appearance. Shortly before death, certain areas of the insect's integument may become darkened. By this time the animal is usually very flaccid and somewhat wrinkled in appearance, and much of the normal plumpness of the body is absent. In general, the insect dies of the disease anywhere between

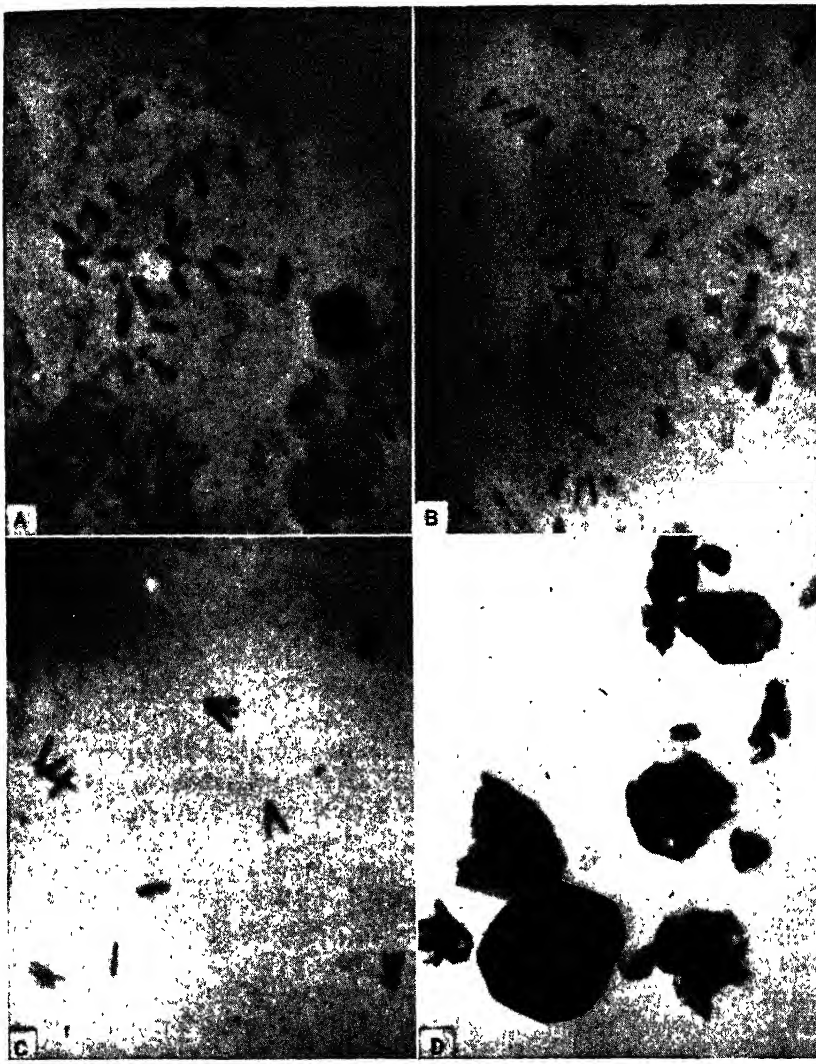


Fig. 152. Electron micrographs of the virus responsible for the polyhedrosis of the alfalfa caterpillar, *Colias philodice eurytheme* Boisd. Magnification approximately 12,000×. *A*. Virus bundles consisting of several virus particles. *B*. Field showing individual virus particles, compact virus bundles, and loosely aggregated virus bundles. *C*. Virus bundles in process of breaking up. *D*. Polyhedral bodies partially dissolved in a weak solution of sodium carbonate, showing the location and position of what are or were apparently the virus bundles. (*Photographs A–C by K. M. Hughes, D by H. B. Wasser and K. M. Hughes.*)

5 and 10 days, usually about 7 days, after infection. During the winter months in the laboratory some individuals are frequently observed to survive 3 weeks after infection.

Soon after the caterpillar dies, the cadaver assumes a "wilted" or "melted" appearance and breaks down into a disintegrating, decaying mass. The dead insects usually remain attached to the alfalfa, frequently hanging by their prolegs. In fields through which an epizootic has just passed thousands of "wilted" larvae may be seen hanging from the plants. With the slightest disturbance the integument breaks and a thick, dark-colored fluid is liberated. Eventually the insect dries down to dark, shriveled, rather brittle remains. Pupae may also show symptoms of the disease, but the somewhat rigid pupal case usually prevents the "wilting" of the insect. Infected pupae are usually darker in color than are uninfected individuals, and at first are frequently mottled with dark and light areas. Adult insects never emerge from pupae which have become markedly darkened. Although the virus may be associated mechanically with the adults and polyhedra may occasionally be found to have been carried over from very lightly infected larvae and pupae, the butterflies are not known to succumb to the infection. The eggs may be contaminated with the virus by the female butterfly, but it is believed that this represents external contamination only and that the egg itself is not infected. No species of insect other than the alfalfa caterpillar has been found to be susceptible to the virus of this lepidopteran.

Longitudinal and cross sections of a diseased caterpillar show polyhedra prominently present in the cells of the hypodermis, adipose tissue, and trachael matrices. (Perhaps one of the earliest microscopic signs of the disease is evident in the hemocytes or blood cells of an infected larva. Smears made of the blood of a caterpillar in the earlier, as well as later, stages of the disease frequently show the nuclei of the leucocytes to contain polyhedra.) In sections treated with picric acid and then stained with iron hematoxylin the polyhedra stain a dark purple or almost black. The nuclei of the infected cells are hypertrophied to a greater or lesser degree, apparently depending upon the number of polyhedra contained within the nuclear membrane. The size of the polyhedra within any given nucleus is fairly constant, although there may be considerable variation between the sizes of the polyhedra in different cells of the same tissue.

The usual size of the polyhedra varies from 1.0 to 3.0 microns in diameter. Exceptionally large polyhedra (4.0 to 5.0 microns) are seen occasionally; the significance of these forms is not clear. Ordinarily the polyhedra show 3 to 6 sides, although they vary greatly in shape. The corners are angular but somewhat rounded. The polyhedra themselves are never round or spherical. They stain feebly or with difficulty with

most aniline dyes. They may be differentiated from fat droplets and from urate crystals by their shape and by the fact that unlike fat droplets they are insoluble in ether and xylol and do not stain with Sudan III, and unlike most crystals seen in insects the polyhedra are not optically active when viewed with polarized light. They are insoluble in water and alcohol but are soluble in acids and alkalies. Since the polyhedra of the alfalfa caterpillar polyhedrosis, like those of the silkworm polyhedrosis, in all probability consist largely of nucleoprotein, it is not surprising that they stain yellow with picric acid, indicating the presence of protein in their make-up.

Histological sections prepared from larvae in advanced stages of the disease or from larvae at the time of death show the vast amount of cellular destruction that accompanies the disintegration of the insect's tissues. The size and number of polyhedra in the nucleus of the infected cell have increased to such an extent that the greatly hypertrophied nucleus occupies almost the entire space of the cell or has burst and liberated the polyhedra into the extracellular spaces. As the cell walls are broken down, large masses of polyhedra accumulate in the space occupied by the tissue, and are also shed into the general coelomic cavity. Several stages of this process are shown in Fig. 153.

Michelbacher and Smith (1943) made numerous observations of this disease as it appeared in the field. They report that although the disease is one of the most important of the natural checks on the insect, in many instances the beneficial action of the disease does not come into play until the alfalfa crop is seriously damaged. Once started, however, the disease can, under favorable conditions, destroy the caterpillar population in an extremely short time. To illustrate this, they cite the following example: On June 29, in one field of alfalfa, approximately 14,000 larvae were collected per 100 sweeps. At this time the wilt disease was just beginning to appear. Three days later, hundreds of thousands of dead caterpillars were observed clinging to the alfalfa, and only 40 live insects were collected per 100 sweeps. Many of these were in the early stages of infection, as were those collected on August 6, when, with every 100 sweeps, only 13 larvae were collected.

In the opinion of Michelbacher and Smith, the two most important conditions for an extensive outbreak of the disease are high humidity and a large host population. Timely irrigation will aid the moisture requirements. Under some conditions, however, the disease does not reach epizootic proportions and instead proceeds at a reduced rate but sufficient to kill enough caterpillars to enable the alfalfa to grow and produce a commercial crop. In the spring of the year the disease is rarely in evidence, but as late summer and early fall approach, more and more

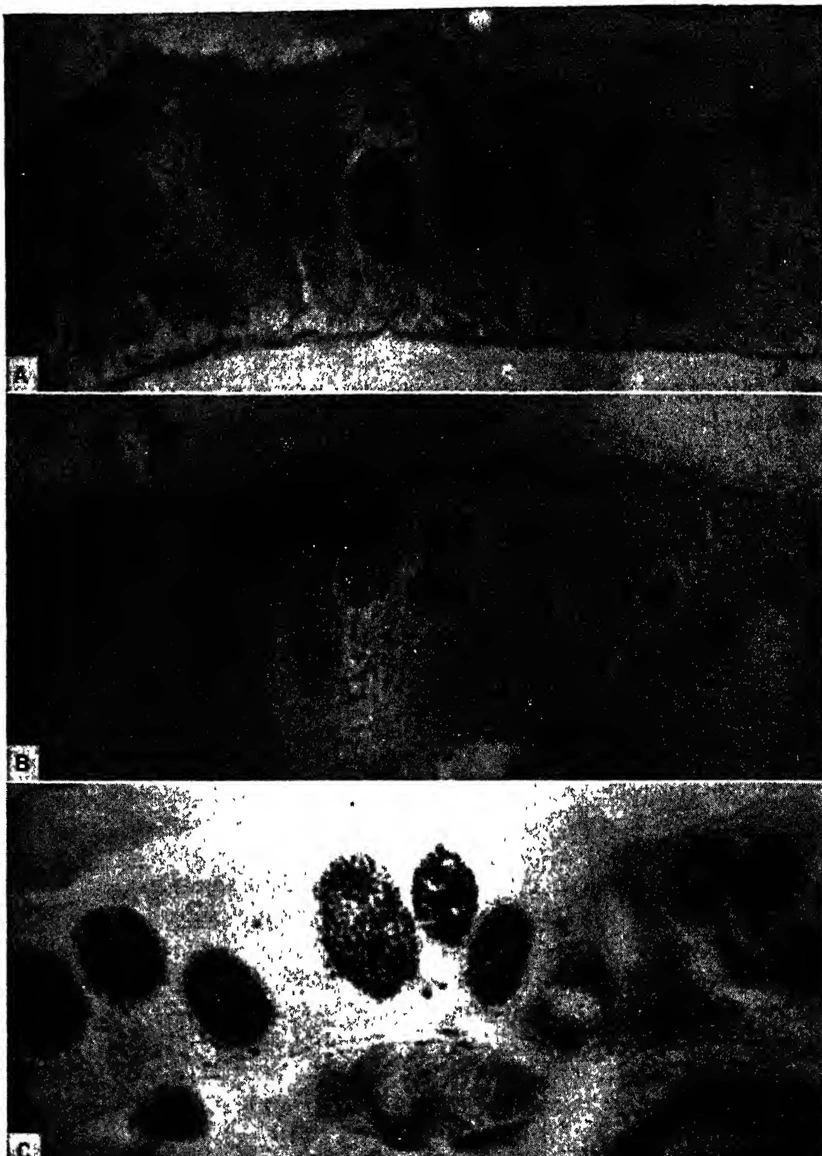


Fig. 153. Progressive stages in the development of polyhedra in the hypodermis of the virus causing this polyhedrosis. Cross sections stained with iron hematoxylin; cell; early stage of infection. *B*. The nuclei of at least two cells showing polyhedra. and cells breaking down. *F*. Still more advanced stage of infection. Cells have been

the caterpillar of the alfalfa butterfly, *Colias philodice eurytheme* Boisd., infected with polyhedra are the dark-staining bodies. *A*. Polyhedra in nucleus of a single hypodermal *C* and *D*. Intermediate stages of infection. *E*. Advanced stage of infection. Nuclei almost completely broken down. (*Photographs by K. M. Hughes and J. M. Smith.*)

infected insects are seen, until as the larval population increases, widespread epizootics may occur.

Field tests in which virus suspensions were distributed artificially on alfalfa fields threatened by the caterpillar have produced encouraging results. In general the results of these tests give the following indications: (1) The virus, applied as a spray, is capable of causing infection in the alfalfa caterpillar and markedly reducing populations, at least in small experimental plots. (2) It is possible to initiate an epizootic of the disease in populations of low density (20 to 30 larvae per 100 sweeps), and that even these low populations can be substantially reduced by the artificial dissemination of the virus. (3) It is possible to initiate an epizootic of the disease in a population of caterpillars earlier than it would occur naturally, thus curtailing the amount of damage done the crop by the insect. (Steinhaus and Thompson, 1949.)

One interesting aspect that requires attention when the use of the virus for purposes of control is considered deals with the effect of the disease on the species of *Apanteles* (*A. medicaginis* Mues.) that parasitizes the alfalfa caterpillar. Michelbacher and Smith noted that the hymenopterous parasite does not seem to be adversely affected in fields where the disease is present but not abundant. The smaller larvae may be parasitized by *Apanteles* while the large caterpillars may be killed by the virus. On the other hand, in fields where the disease is destroying large numbers of the larvae the number parasitized by *Apanteles* is often less than it is in the surrounding fields. It is possible that the parasitized caterpillars are killed by the virus before the parasite can complete its development. Instances have been observed, however, in which *Apanteles* has dominated the situation after the disease had first reduced the number of larvae and no subsequent increase in the larval population resulted. It is possible that the *Apanteles* parasites may transmit the disease through the contamination of their ovipositor, though this remains to be proved.

Other species of *Colias* may also be subject to virus diseases and, in fact, the first recorded instance of this type of disease in a species of *Colias* may be that in the lucerne caterpillar, *Colias electo* Linn., in South Africa (Mally, 1908; Lounsbury, 1913*a*; Smit, 1936). Mally reported this as a bacterial disease, but indications are that it is of the type we now know to be caused by a virus. Lounsbury describes attempts to utilize the disease as a means of control in the field but attributes most of the success obtained to the natural presence of the disease. He also mentions concurrent tests against the pepper-tree caterpillar, *Bombycomorpha bifascia* (Wlk.), but indecisive results were obtained. Earlier than these reports, however, was that of Edwards (1887), who referred to the destruction of

larvae and chrysalids of *Colias hagenii* Edw. by a disease that might possibly have been a polyhedrosis.

The imported cabbageworm, *Pieris rapae* (Linn.), apparently suffers from a polyhedrosis, but the actual demonstration of polyhedra does not seem to have been reported. Several authors mention the presence of disease in this insect, but it is not always clear that the infection referred to is one caused by a virus. Pospelov and Noreiko (1929) were probably concerned with such an infection in their work. Although Brown (1930) considered the disease he studied to be caused by a bacterium he might actually have been dealing with a virus infection. Kawada and Sekiya (1940) also refer to a disease of this insect. Richards (1940) found considerable mortality of the larvae due to "wilt" under laboratory conditions, but he thought that the incidence would be lower under field conditions. In Hawaii, Holdaway *et al.* (1941) observed large numbers of the caterpillars to be affected by a "wilt" in nature, especially at elevations below 1,000 feet. In many places where the disease was present, the larvae were not parasitized by *Apanteles glomeratus* (Linn.), while at high elevations parasitism reached its full height and the disease was not found. When their hosts were infected with the virus, the larvae of the parasite were observed to die. On the other hand, larvae of a tachinid fly (*Frontina archippivora* Will.), if well developed when the host succumbs to the disease are able to emerge in an almost normal fashion.

The cabbage butterfly of Europe, *Pieris brassicae* (Linn.), is subject to a virus disease which, however, is not characterized by the formation of typical polyhedra. Instead peculiar refringent bodies of very irregular form are present in the blood of diseased larvae. Since this "inclusion disease" is different from those we are discussing at present, it will be considered at a later point in this chapter.

Nymphalidae. In August, 1935, Paillot (1935*b*, 1936) discovered a polyhedrosis of *Vanessa urticae* (Linn.) in the Rousses region of France, near the French-Swiss border. The epizootic was severe, most of the larvae being unable to become chrysalids. The outbreak was rather localized since a few miles away the larvae were healthy. In the early stages of the disease, Paillot found it difficult to distinguish the sick from the healthy larvae. The dead larvae, however, liquefied very rapidly.

The blood of a heavily diseased caterpillar is milky turbid in appearance, and the hemocytes contain polyhedra analogous to those in gypsy-moth wilt. The shape of these bodies apparently is not very regular or well defined, since more or less square, triangular, polygonal, and angular rounded forms may be seen. The polyhedra appear only in the hemocytes and in the cells of the hypodermis, fat tissue, and tracheal matrix, and

sometimes in the genital capsule. The virus infects chiefly the nucleus and destroys the chromatic substance and nucleolus.

The disease is very contagious, and experimentally it may be transmitted by the digestive route or by inoculations directly into the body cavity. Transovarial transmission is probable but has not been demonstrated with certainty.

Collier (1934) has described a polyhedral infection of *Argynnis lathonia* Linn. The size and shape of the polyhedra were similar to those found in nun-moth caterpillars suffering from *Wipfelkrankheit*. A bipolar bacterium, similar in morphology to those of the *Pasteurella* group, is a secondary invader in the disease as observed by Collier.

Polyhedrosis of the European Spruce Sawfly (Hymenoptera)

In 1930 the European spruce sawfly, *Gilpinia hercyniae* (Htg.), was discovered to have caused considerable defoliation of spruce in the province of Quebec, Canada. By 1938 the infestation had reached its peak, after which the number of insects declined until by 1940 no great damage was caused. This decline coincided with the appearance of a polyhedrosis of the sawfly larvae, and the evidence, as presented by Balch and Bird (1944), indicates that this disease was responsible for the great reduction of the population of this destructive insect.

Balch and Bird, who have outlined the early history of the disease, relate that the first indication of a disease affecting the sawfly was seen in 1936 in insects reared in the laboratory by C. C. Smith. For 25 generations (1934–1935) there had been no signs of disease in the stock animals, but early in 1936 small percentages of the larvae began to die. The amount of mortality increased, until by 1939 it was impossible, by ordinary methods, to rear the larvae in the laboratory.

Up to 1938, individual larvae that might have been diseased were seen in the Canadian forests only on rare occasions. About this time, diseased larvae were observed in some parts of New Brunswick. In 1937, infected larvae were noticed occasionally in southern New Hampshire and Vermont, and in 1938 the disease was common in these localities (Dowden, 1940). The disease spread to wider areas and apparently was responsible for controlling the insect in these two states. About this same time the disease was beginning to appear in Maine, but here it was not considered as a reliable factor of control (Peirson, 1941). During the period from 1939 to 1942 the epizootic apparently spread to Canada from south to north. It was first reported on the north shore of the St. Lawrence River in 1940 (Daviault, 1941). By 1942 it occurred throughout the range of the sawfly from Nova Scotia to Lake Ontario. Although at first it was observed principally in heavily infested areas, it was soon found causing

significant mortality in areas of light infestation. According to Balch and Bird, it appears that a high density of host population may have been necessary, or at least favorable, to the development of the epizootic but that it rapidly achieved a momentum that carried it long distances more or less regardless of the density of the population of the insect.

As to the original source of the causative virus, there is no certainty. It may have been introduced into North America along with imported insect parasites. A similar disease affects *Gilpinia polytoma* (Htg.) in Europe.

Fig. 154. Larvae of the European spruce sawfly, *Gilpinia hercyniae* (Htg.), suffering from polyhedrosis. (*Courtesy of F. T. Bird.*)

One of the most complete reports on the infection in *Gilpinia hercyniae* (Htg.) is that by Balch and Bird (1944), to which we have already referred. Much of the discussion that follows is based on their findings.

Symptoms. One of the first symptoms of the disease is the appearance of a faint yellow discoloration of the third to fifth abdominal segments of the insect, which is normally of a distinct green color. This is noticeable particularly among the third-, fourth-, and fifth-instar larvae; in the first and second instars there is a similar whitish discoloration, but this is not easily recognizable in larvae of such a small size. The discolored area becomes more pronounced, until the entire larva changes from its healthy green color to a yellow-green shade, and after death to a dark brown or black. The infected larva loses its appetite, ceases to feed, and becomes shortened as though the insect had been starved. Sometimes the animal exudes a dark-brown fluid from the anus which "glues" the cadaver to the needle on which it has been feeding. The yellow-green protective fluid that a healthy larva emits from its mouth when it is disturbed has a milky-white appearance in the case of an infected larva. When the insect dies, it is usually completely flaccid, and attempts to remove the

cadaver from the foliage rupture the integument, liberating the liquid contents, which have no offensive odor. After being dead for some time, the larva appears shriveled, wrinkled, and dry.

Balch and Bird found that the period from infection to the appearance of the first external symptoms varied with the temperature at which the larvae are reared. At 21°C. this period is of about 4 days' duration, with death occurring 2 or 3 days later. At a mean temperature of approximately

Fig. 155. Photomicrograph showing parts of the midgut, a Malpighian tube, the hindgut, and the gut lumen of a larva of *Gilpinia hercyniae* (Htg.) infected by virus. Stained with hematoxylin and eosin. The polyhedral bodies are deeply stained and may be seen in the hypertrophied nuclei of the midgut epithelium. The Malpighian tube and hindgut epithelium do not appear to be susceptible to infection. (*Courtesy of F. T. Bird.*)

19°C. the period from infection to death was found to be 6 days, and at about 10°C. this period was 11 days.

Pathology. If the body content of a diseased sawfly larva is examined microscopically, it will be found to contain the usual types of disorganized tissue and debris together with large numbers of highly refractive polyhedral bodies. The polyhedra range from 0.5 to 1.8 microns in diameter and have an average diameter of 1.3 microns. Their shape is also variable but is usually that of a polyhedron with corners somewhat rounded, though it is never perfectly spherical. As with other polyhedra, they resist staining. An ultramicroscopic virus is undoubtedly present, but so far it has not been shown to be filterable. In some experiments it has been found that Berkefeld V and N filter candles retain the virus.

Of particular interest from the pathological standpoint is Balch and Bird's (1944) assertion that the pathological process is concerned with the

digestive tract and usually results in complete destruction of the midgut. The cells of the mesenteron epithelium become enlarged, and within the hypertrophied nuclei the polyhedral bodies are formed. When examined in the fresh state with a low-power lens, these nuclei appear as small dark bubbles. Eventually the cells break down and the polyhedra escape. In healthy larvae the intestinal epithelium is translucent and, because of the food it contains, the gut appears green in color. In diseased larvae, on the other hand, the epithelium becomes opaque and milky-white in color, and the gut contains no food. These changes in the intestinal tract may take place very rapidly—within 12 hours. Now this picture is very much unlike that which occurs in most other polyhedroses where the principal tissues affected are the fat body, hypodermis, tracheal matrix, and blood cells, while the intestinal epithelium is one of the last tissues involved and even then rarely gives rise to polyhedra.

Another interesting pathological change is the formation of tumors in the diseased sawfly as the result of the polyhedrosis. Stimulated by the activity of the virus in the cells of the midgut, the regenerative cells, or nidi, proliferate to the outer, or coelomic, surface of the gut, where eventually the tumor may completely surround the intestinal tract (Bird, 1948).

When the feeding larvae are infected too late to be killed before the cocoon is formed, the stages of the cocoon, as well as of the adult, show internal evidence of the disease, as determined histologically (Balch, 1946). Such adults, however, show no external symptoms, and the female may retain her ability to lay eggs.

Epizootiological Factors. Transmission of the virus from a diseased to a healthy insect apparently occurs principally by way of contaminated food. Larvae are easily infected by allowing them to feed on foliage that has been smeared or sprayed with water in which material from diseased insects has been suspended. Other methods of transmission probably occur. There are indications that under certain conditions the virus may be carried into the cocoon without killing the insect, but the emerging adult is contaminated. Balch and Bird suggest that this may be an important means of spreading as well as overwintering the virus, since the adults often fly long distances. It is not known for certain if the virus overwinters on the foliage. Since most of the cadavers are washed off before spring, the trees are probably fairly well cleansed. Also it is known that contaminated foliage may lose its infectiveness during a month of subzero weather. On the other hand, the Canadian workers found the pathogen to survive in cadavers stored at just below the freezing point for 13 months and in aqueous suspensions at room temperature for at least 3 months. No change in the virulence of the virus in the field has been noted over a 10-year period. Although the disease is highly con-

tagious, it does not appear to spread through the air except as it is carried in water or on dust particles that have been in contact with the virus. That the virus may be passed through the egg has not yet been demonstrated.

Gilpinia hercyniae (Htg.) has five feeding larval stages during which it is susceptible to infection. The sixth stage, the prepupal period, the pupa, and the adult do not appear to be susceptible. If the period between the infection of the fifth-stage larva and the usual evacuation of the gut by the sixth stage is less than the period of incubation for the disease, normal development to the adult stage can take place; *i.e.*, if the insect acquires the virus late enough in its larval life, the disease will not have progressed far enough to be disastrous by the time it becomes an adult. On the other hand, Balch and Bird point out that if the time of infection of a fifth-stage larva is such that lesions occur in the gut shortly before molting, the insect may reach the sixth stage but be unable to evacuate the gut. A cocoon may be formed, but death takes place in the eonymph or occasionally in the pronymphal stage. In such cases the dead larva is dark and flaccid in appearance and the cocoon is rather loosely spun. No cases of individual immunity or resistance have been observed in any of the feeding larval stages.

One of the most important factors in determining the extent and severity of an epizootic is the density of the sawfly population. The greater the density of the population, the higher is the relative mortality. Light infestations of the insect, however, are affected (Peirson, 1942, says that the virus is equally effective where the population is light, and Bird, 1947, reports that the disease is an effective control agent at low levels of sawfly populations), but the percentage of diseased insects increases with the numbers of its host. This increase appears to be independent of secondary effects of crowding, such as a limited food supply. Furthermore the disease does not completely eliminate the insect from an area, and it tends to disappear while a few uninfected larvae still remain. The probability that there is a minimum level of population on which the polyhedrosis can maintain itself has been suggested.

According to Bird (1947), local cycles of sawfly population have been observed as a result of the disappearance and reappearance of the disease. In one plot, for example, the population increased 20-fold within a 2-year period following the disappearance of the disease at a low level, but in the third year it was reduced again to the low level. In general, similar cycles occur at the same time in areas having similar climates. The activities of introduced insect parasites affect the cycle by limiting the rate of increase when the polyhedrosis is absent or at a low level.

Although there are indications that the virus is more destructive in lower altitudes, there is little evidence to indicate that local weather con-

ditions greatly influence the disease. More observations on this are necessary, however, since temperature and humidity are probably very important factors in the epizootiology of the disease.

Value of Disease in Control of the Sawfly. There appears to be little doubt that in Canada the polyhedrosis we are discussing has been of major importance in the control of the European sawfly. Balch and Bird (1944) have presented convincing evidence of this in spite of the fact that exact measurement of the percentage of insects killed is difficult. By recording daily the stage and condition of the larvae dropping from the trees on 2- by 2-foot trays, these workers were able to obtain significant data for estimating the percentage of mortality from 1938 to 1941 in New Brunswick. Balch and Bird decided that the annual reduction in larval populations is best indicated by the number of larvae reaching the sixth stage in a healthy condition. This stage does not feed and hence does not become diseased unless infected in an earlier instar. After remaining on the tree 1 or 2 days, it normally drops to the ground and spins its cocoon.

Figure 156 shows the numbers of fifth-stage larvae that dropped from the trees in one of the Canadian plots during 3-day periods from 1939 to 1941. The graph indicates at least two things: (1) it shows the increasing effectiveness of the disease as the summer progresses; (2) it gives some idea as to the degree of mortality and the reduction in population each year. An example of the actual numbers obtained in the dropping of sixth-stage larvae may be cited: on one plot, for instance, the total dropping in 1938 was 1,389 larvae; each of the following 3 years the number was 465, 19, and 2 larvae. Balch and Bird calculate that the percentage mortality on this plot ranged from 94.8 per cent 1 year to 99.7 per cent the next 2 years. The extent of larval mortality is also reflected in the overwintering population of cocoons, a marked decrease occurring following seasons of high larval mortality. When the mortality from disease was compared with the total mortality (*i.e.*, mortality from all causes), the importance of the disease in bringing about the end of the sawfly outbreak was still more apparent.

In 1946 Balch reported that although it was still common, the sawfly had remained at a fairly low level of population and had caused no noticeable damage. This excellent state of affairs was attributed to the effects of the disease and insect parasites. He also mentioned that dried extract of diseased larvae had been used to establish the disease in Newfoundland, where previously no diseased larvae had been seen. The disease soon became prevalent over considerable areas surrounding the points of liberation.

In the infested region of northeastern United States similar, but not so

complete, observations have been made on the natural control of the insect by the polyhedral disease. In Vermont and New Hampshire, Dowden (1940) observed a high degree of mortality in areas of both heavy and

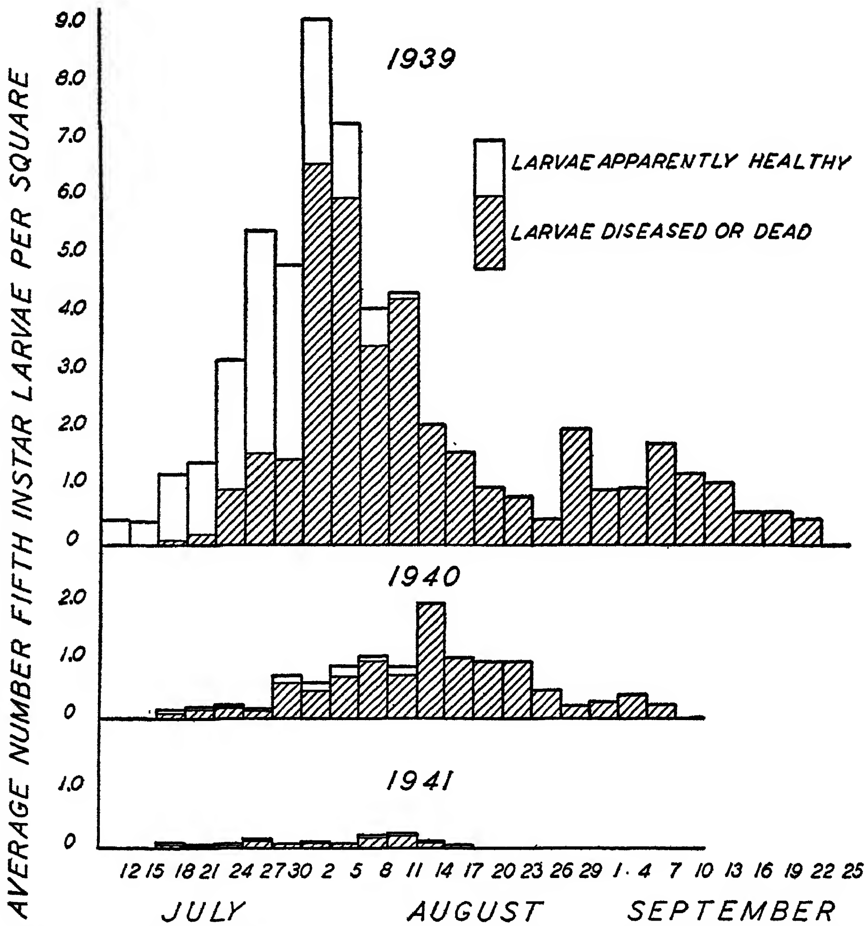


Fig. 156. Numbers of fifth-instar larvae of the European spruce sawfly dropped from spruce trees in a Canadian plot (1939–1941), showing as apparently healthy or diseased. (*From Balch and Bird, 1944.*)

light infestations. The number of dead larvae was small at the beginning of the season, but toward the end the percentage of mortality had greatly increased. In areas of heavy larval concentrations, the insects would fall to the ground and congregate in large masses at the bases of the trees. Practically 100 per cent of the larvae in such masses apparently died from the disease.

In Maine, Peirson (1942) thought that the disease was probably the most important control factor—destroying up to 70 per cent of the larvae in some areas. Dirks (1944), also working in Maine, observed the disease to reduce greatly the sawfly population of 1941 and 1942. In some experimental plots he found the percentage of diseased larvae to range between 70 and 99 per cent.

Polyhedroses of Other Hymenoptera

In addition to *Gilpinia hercyniae* (Htg.) and *Gilpinia polytoma* (Htg.), other sawflies (superfamily Tenthredinoidea) have been reported as susceptible to polyhedral diseases. Balch and Bird (1944) mention *Gilpinia pallida* (Klug.), *Diprion pini* (Shrank), and *Neodiprion sertifer* (Geoff.). Others are *Diprion rufus* Ratz, *Nematus erichsonii* Hartig, and, depending upon the interpretation given the inclusions described by Heidenreich (1939), possibly *Pamphilius stellata* Chris.

An epizootic disease was observed in Minnesota by Graham (1925) to break out suddenly in a population of the jack-pine sawfly, *Neodiprion banksianae* Roh., almost wiping it out. The disease outbreak occurred at a time of high humidity. Polyhedra were not demonstrated at the time, but from the available information there is little doubt that a virus was the causative agent. A similar disease may have been observed in the black-headed sawfly, *Neodiprion abietis* Harris, which attacks balsam and spruce trees.

Polyhedroses of Diptera

The first dipteran insect reported as suceptible to a polyhedral disease was the bluebottle fly, *Calliphora vomitoria* (Linn.) (see Chapman and Glaser, 1915), but this observation needs confirmation. In 1923 Rennie described a polyhedral disease in larvae of *Tipula paludosa* (Meigen) in Scotland. Although similar to the polyhedral diseases of Lepidoptera, in some respects it appears to be quite different.

Except in an advanced stage, the *Tipula* larvae show no distinct signs of infection. As the disease progresses, however, the normal "earthy" color of the larvae becomes pallid and finally appears chalky white. The blood becomes a milky-white fluid that flows out readily when the insect's integument is pricked. Microscopic examination shows the fluid to be filled with large numbers of irregularly shaped, colorless, translucent, highly refractive bodies. Also to be seen are numerous detached fat cells containing polyhedral bodies.

The polyhedra are heavier than water and are insoluble in alcohol, ether, chloroform, glycerin, benzene, or hydrogen peroxide. In 1 per cent sodium hydroxide they swell to double their volume and then appear

to have a finely granular core. In ammonia they differentiate into an inner mass and a peripheral layer. Acetic acid also causes them to swell, and in such preparations they appear to have a granular interior with a clear surface region. As with other polyhedra, osmic acid does not blacken them nor do they stain with Sudan III, thus indicating the absence of fat.

As concerns the histopathology, Rennie (1923) states that the nucleus of an infected fat-body cell shows progressive hypertrophy as the infection

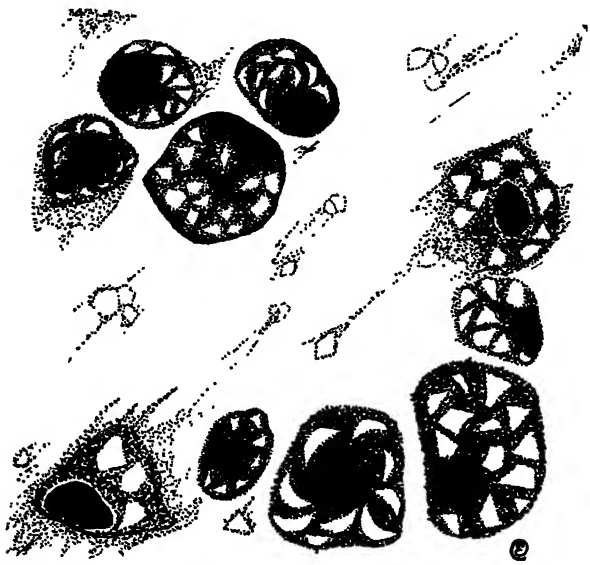
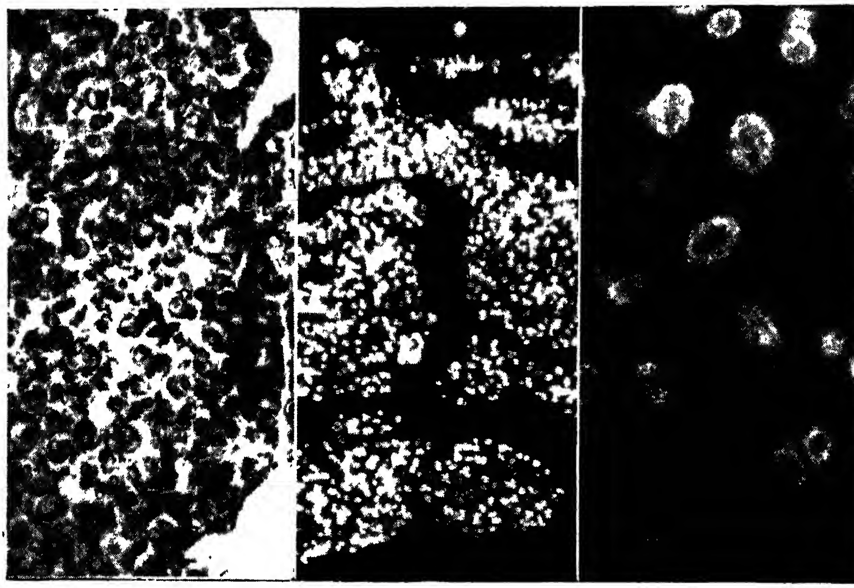


Fig. 157. Polyhedral bodies characteristic of the polyhedrosis of the larva of *Tipula paludosa* (Meig.), as they appear in the hypertrophied nuclei of fat cells, the cytoplasm of which has largely disappeared. The dark areas in the nuclei represent chromatin masses. (*Drawn from photograph by Rennie, 1923.*)

proceeds and that the following stages can be noted: (1) The nuclear chromatin is gathered in granules that form grapelike clusters surrounded by a clear ring. These granules are later found on the periphery of the nucleus. The cytoplasm consists of but a thin layer. (2) A chromatoid mass or masses, sometimes two or more in number and rounded in form, appear in the body of the nucleus. The remainder of the nucleus is granular in appearance, and the cytoplasm has almost disappeared. (3) The polyhedra appear symmetrically in a ringlike form on the surface of the central mass. They are usually triangular in shape and are sharply angled; sometimes they appear regularly crescentric, resembling the segment of an orange. (4) The polyhedra then become massed in one hemisphere of the nucleus. At this stage their shape is more or less ovoid, but they

never become symmetrical. (5) Finally, the hypertrophied nucleus bursts, liberating the polyhedra into the blood.

Very few additional species of Diptera are known to be susceptible to polyhedral virus diseases. *Aporia crataegi* Linn. was observed, between 1921 and 1924, to suffer from intermittent outbreaks of a polyhedral



A B C

Fig. 158. The inclusion disease of *Camptochironomus tentans* (Fabr.). *A*. Section through an infected larva showing the ovoid inclusions in the fat body. The dark-staining nuclei are more compact than normal. *B*. Dark-field view of ovoid inclusions in fat tissue, at about one-half the magnification of *A*. *C*. Dark-field view of ovoid inclusions at a high magnification, showing the minute granules contained in the inclusions. (*Courtesy of J. Weiser.*)

disease that covered wide areas of the Rhine Palatinate (Stellwaag, 1924), and Martelli (1931), in Italy, mentions that the population of this insect in the areas he studied was considerably reduced by a polyhedrosis. Doeksen (1938) reports that polyhedral diseases were seen in larvae of two species of wheat gall-midges, *Contarinia tritici* Kirby and *Sitodiplosis mosellana* Géhin.

Inclusion Disease of Chironomids. At this point may be mentioned a peculiar disease found affecting the larvae of *Camptochironomus tentans* (Fabr.) in Europe. Weiser (1948) refers to the infection as a "polyhedral disease," but it differs from most known polyhedroses in several interesting

ways. The infection appears to be centered in the fat tissue of the insect. Within the cytoplasm, but not the nucleus, of the infected fat cells appear ovoid to octahedral inclusion bodies ranging in size from 2 to 16 microns in diameter (Fig. 158*A*,*B*). The nucleus of the host cell does not seem to be adversely affected, nor does it become hypertrophied. With a dark-field microscope the ovoid bodies appear to be filled with very minute granules about 0.1 to 0.2 micron in diameter (Fig. 158*C*). The ovoid inclusions and the granules are soluble in acids and alkalies (pH 1 to 3 and pH 9 to 13).

Infected larvae become inactive, cease feeding, and usually die in 7 to 14 days or at the time of pupation. The fat body of the infected insect can be seen as a whitish mass showing through the integument.

It is probable that this disease represents a heretofore unrecognized group of virus agents. When this and probably other examples of the same type of agent are further studied, it may be convenient to place them in a separate generic group as we have done for the other insect viruses.

Possible Polyhedroses in Other Insect Groups

According to von Prowazek (1907), Bolle was able to infect larvae and adults of the larder beetle, *Dermestes lardarius* Linn., with the virus of silkworm jaundice, although the form of the polyhedra was somewhat different in shape from that of the original. Chapman and Glaser (1915) include in their list of virus-susceptible insects reported by European authors another dermestid, *Anthrenus museorum* Linn. The larva of *Celosterna scabrator* Fabr., when reared in the laboratory, has been reported by Beeson (1931) in India to be susceptible to attack by a "wilt disease," possibly of virus origin. A fungus frequently appeared as a secondary invader.

It is difficult to know just how to appraise these observations. Bolle's report on the susceptibility of the larder beetle to the silkworm virus appears especially questionable. The polyhedra he observed may have represented part of the original inoculum, the shape of the bodies changing somewhat in form in their new environment—but one can only speculate on this possibility. Furthermore, since adult insects generally are not susceptible to polyhedroses of their corresponding larvae, it seems unlikely that Bolle was working with a frank infection in the case of the adult *Dermestes lardarius* Linn. No sound well-authenticated report of a virus infection in a coleopterous insect has yet been made. The same may be said of all orders of Hexapoda other than those we have discussed (Lepidoptera, Hymenoptera, and Diptera).

It is possible that diseased insects having the general symptoms of a polyhedrosis may actually be infected with a nonvirus agent. Spencer

(1945) reports a disease of the giant willow aphis, *Pterochlorus viminalis* (Fons.) (Homoptera), in which the aphids are literally liquefied into black drops that fall to the ground. The same or a similar disease has been observed in this insect in southern California by Essig (1929). Slide specimens of some of the latter, however, have been examined by the author and have been found to contain peculiar resting spores or conidialike bodies, indicating a pathogen of probable fungous nature. Similar infections have been noted in *Macrosiphum ambrosiae* Thos. from Illinois, and in *Lachnus persicae* Cholodk. from Palestine.

VIRUS DISEASE CHARACTERIZED BY THE PRESENCE OF REFRINGENT POLYMORPHIC INCLUSIONS

In 1924*b*, Paillot described an interesting but somewhat puzzling type of virus disease of the larva of the cabbage butterfly of Europe, *Pieris brassicae* (Linn.). The pathological characteristics of the infection were such that it could not be considered a polyhedrosis in the usual sense of the word. Instead it was characterized by peculiar refringent bodies of very irregular form present in the blood and in certain cells of the diseased larvae.

The disease is apparently a common one in France, at least during certain years, and it frequently plays an important role in the natural control of the insect (Paillot, 1926*d*, 1943). In certain localities the mortality due to this disease reaches significant proportions. It is not as destructive, however, as the polyhedroses. High humidities and warm temperatures enable the disease to develop more rapidly than do moderate degrees of these factors. In contrast to such polyhedroses as jaundice of the silkworm, this disease develops well at temperatures below 18°C., its development being suspended at temperatures below 8°C.

Externally there is nothing definite by which the diseased larvae may be distinguished from the healthy ones. The blood of the former is viscous and milky in appearance and on microscopic examination may be seen to consist of morphologically altered blood cells and the peculiar refringent bodies characteristic of the infection. When the blood is subjected to ordinary centrifugation, the blood cells and the refringent bodies are sedimented out, but the supernatant remains cloudy. If this supernatant is examined with a dark-field microscope, it shows the presence of numerous feebly lighted granules, similar to those which occur in silkworm jaundice. These granules, less than 0.1 micron in diameter, are found in large numbers in vacuoles and in the fluid parts of the cytoplasm of the micronucleocytes (leucocytes). The granules are retained by Chamberland filters of fine porosity, and the virulence of the blood is thus destroyed. Heating infectious blood to 70 or 72°C. for ½ hour diminishes its virulence con-

siderably. Held at 75°C. for the same length of time, the blood loses its virulence completely. Paillot (1926*c*) gave to the granular elements, which he considered to be the active causative agent of the disease, the name *Borrellina* [*Borrelina*] *pieris*. Since the virus of this disease has not yet been demonstrated by the electron microscope, the true significance of the granules described by Paillot and their possible relation to the virus particle itself have not been ascertained. Indeed such relationships have not been determined even in the case of silkworm jaundice, in which, although the rod-shaped virus particles have been seen by the electron microscope, their relation, if any, to the granules described in this insect by Paillot remains uncertain. In any case, following the concept outlined at the beginning of this chapter, the virus of the pierid disease under discussion may be designated as *Paillotella pieris* (Paillot).

The virus apparently multiplies only in the cells of the fat tissue and in the micronucleocytes (leucocytes) and oenocytoids of the blood of the caterpillars. When larvae are experimentally infected, the cellular lesions usually appear within 24 hours. The granulated chromatin has a tendency to condense into somewhat irregular masses. The nucleus itself loses its individuality, and close beside it appears a diffused mass that stains light pink with Giemsa's solution. Later, within the cytoplasm of the cell, there forms an equatorial ring, easily visible in fresh preparations. The ring always crosses the diffuse mass and appears to be in direct contact with it. Eventually the cell is destroyed, and the refringent rings float free in the hemolymph. Not all the bodies that float in the blood are in the form of rings; they take numerous irregular shapes and sizes. Although they are readily visible in fresh preparations, they are not demonstrable in stained smears. The infected blood cells, particularly the oenocytoids, frequently form giant cells containing several irregularly shaped nuclear areas. In addition to the blood, the inclusions also appear in the cells of the adipose tissue.

These refringent bodies do not originate from the chromatin material of the nucleus, as some believe the polyhedra to do. Instead they appear to arise from the mitochondria of the cell. Using mitochondrial methods of histology, this transformation can readily be seen in the adipose cells. In normal cells the rod-shaped mitochondria are scattered throughout the cytoplasm. In diseased cells the mitochondria concentrate at various points in the cytoplasm and break down into granular elements that tend to fuse. There are thus formed mitochondrial masses (chondriosomes) which appear as true siderophilic inclusions. This is a rather transitory stage; the refringent bodies arise by the elongation of these masses. At an advanced stage these bodies no longer stain except at their peripheries. Just what determines their various sizes and shapes is not clear.

Experimentally, at least, the virus of this inclusion disease is difficult to establish in insects when administered by the oral route. On the other hand, infectious material introduced directly into the body cavity will initiate the disease without much trouble. In nature the disease appears

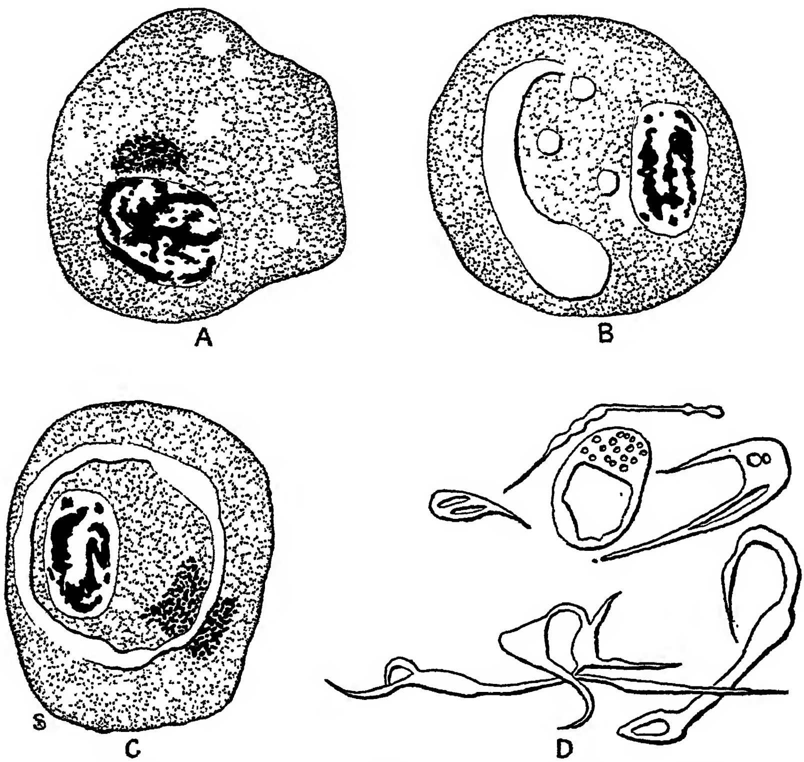


Fig. 159. Manifestations of the refringent polymorphic-inclusion disease in *Pieris brassicae* (Linn.). *A*. Blood cell showing the diffused cytoplasmic mass but no refringent bodies. *B*. Blood cell with refringent bodies. *C*. Blood cell with refringent ring running through the cytoplasmic mass. *D*. Refringent bodies in suspension in the hemolymph of *Pieris*. (*Redrawn from Paillot, 1926d.*)

to be transmitted principally through or along with the egg from one generation to the next.

As in the case of many of the other virus diseases of insects, the pupal stage of the cabbage butterfly may also suffer and die as the result of the infection. It is possible that sometimes the pupa may become an adult butterfly before the virus has had a chance to exert its destructive effect and that such butterflies can harbor and distribute the virus not only via the egg but by mechanical means as well.

This polymorphic-inclusion disease is so far the only one of its kind

that has been described. If, as Paillot believes, the source of the refringent bodies characterizing this disease actually is different from that which gives rise to the polyhedra in the infections they characterize, it might be argued that the activities of the two causative viruses are likewise of distinctly different types. This in turn may indicate at least a generic difference in the two types of virus. In the future, therefore, we might expect to find other examples of the polymorphic-inclusion group.

VIRUS DISEASES CHARACTERIZED BY THE PRESENCE OF GRANULAR INCLUSIONS

(Granuloses, Pseudo-grasseries)

As we have seen, the polyhedron is not the only type of inclusion body found in insects infected by agents generally considered to be virus in nature. As our knowledge of insect viruses has increased, it has been found that the different types of viruses may be characterized by several different types of inclusion bodies which may logically be separated into rather distinct groups. As stated at the beginning of this chapter, in addition to those viruses which are not accompanied by inclusions of any kind, we may also recognize as distinct groups those viruses which cause the formation of polyhedra, those characterized by the formation of refringent inclusion bodies of irregular form and dimensions, and those viruses which are characterized by the presence of certain kinds of "granules" in the infected cells. So far in this chapter we have concerned ourselves with the features of those viruses which are accompanied by the formation of polyhedra and by the formation of irregular inclusion bodies. Let us now turn to a consideration of those viruses characterized by the formation of granules or granulelike elements in the host's tissues.

As it is now constituted, the group of granule-producing viruses is a small one. It is entirely possible that numerous additional examples of this group exist but have gone unrecognized. The first one of the group to have been discovered appears to be that occurring in the larva of the cabbage butterfly of Europe, *Pieris brassicae* (Linn.), and described by Paillot in 1926(*d*). A few years later, in 1934, this same worker reported a similar disease, which he called "*pseudo-grasserie*" in larvae of the cutworm, *Euxoa segetum* Schiff. (common names include turnip moth and the common dart). The next year he found another type of this form of infection in the same insect, and still later, in 1937(*b*), he described a third type in the same host. Paillot considered each of these three types to represent three different infectious agents and designated them "pseudo-grasserie 1," "pseudo-grasserie 2," and "pseudo-grasserie 3." Then, in 1947, Steinhaus reported the first discovery of this general type of infection

in the Western Hemisphere.[1] In this instance the host was the variegated cutworm, *Peridroma margaritosa* (Haw.). A year later, Bergold (1948) observed a similar disease in larvae of the fir-shoot roller, *Cacoecia murinana* Hb., in Europe. Thus it became evident that there exists a group of infections, virus in nature, that are apparently distinct from the polyhedral-virus infections and from those infections in which the viruses are not associated with inclusion bodies.

The name "pseudo-grasserie," or its anglicized form "pseudo-jaundice," appears to be inadequate and misleading. In the first place, the form "pseudograsserie" has been used (Paillot, 1919) as the name of a bacterial disease of the gypsy-moth caterpillar. Secondly, there is no concrete evidence that the agent causing these diseases is related to the virus causing *grasserie* or jaundice of silkworms. It seems expedient, therefore, to make a clearer nomenclatorial distinction between the polyhedroses and the type of infection here under consideration. Accordingly, we shall, for the time being, designate these diseases characterized by the formation of large numbers of granular inclusions in the cytoplasm of the infected cells as "granuloses."

Nature of the "Granules." Upon his discovery of the granulelike inclusions that characterize the type of disease under consideration, Paillot believed these bodies to be of a nature similar to those seen in dark-field preparations of jaundiced silkworms and to be the virus itself. The hyaline inanimate aspect of these bodies, however, indicates that their nature is not so simple. While working with the granulosis of the variegated cutworm, *Peridroma margaritosa* (Haw.) it occurred to the author, in 1948, that the granules might represent some sort of a protein envelope which covered the causative agent, possibly a virus. Electron micrographs showing viruslike particles protruding from the inclusion bodies strengthened this supposition. Strong proof that the virus particle is enclosed in an envelope of protein material came with the demonstration of this fact by Bergold in 1948 while working with a similar disease in the tortricid, *Cacoecia murinana* Hb. According to Bergold, each capsule contains only one virus particle. By treating the capsules with an alkaline solution, the rod-shaped virus particle can be caused to "slip out" of the capsule. Following Bergold's technique the writer and his associates were able to confirm his earlier observations and were able to demonstrate the virus particles in the case of the disease of the variegated cutworm.

Whether or not the relationship between the virus and the granules as demonstrated in the two cases mentioned above is the same for the gran-

[1] As this is written (1948), C. G. Thompson and the author have observed similar granuloses in the salt-marsh caterpillar, *Estigmene acraea* (Drury), and in the buckeye caterpillar, *Junonia coenia* Hüb., in California.

uloses of *Euxoa segetum* Schiff. and other insects is not yet known; in all probability it is.

Granulosis of *Pieris brassicae* (Linn.)

In 1926(*d*), Paillot observed larvae of the cabbage butterfly of Europe, *Pieris brassicae* (Linn.), suffering from a disease that in some respects reminded him of the polyhedrosis of the silkworm known to French workers as *grasserie* (jaundice). For this reason, he referred to the disease in the cabbage-butterfly larvae as *grasserie*. Because further study showed the two diseases to be of distinctly different types, he later (1934) designated the pierid disease as "*pseudo-grasserie*," after giving the associated granular inclusions, which he considered to be the cause of the malady, the name *Borrellina* [*Borrelina*] *brassicae* (1926*c*,*d*). (The etiological agent of this disease may now be designated *Bergoldia brassicae* (Paillot) *comb. nov.*) Thus the term "pseudo-grasserie" more or less grew out of an early belief in the similarity of the disease to silkworm *grasserie*. This is one of the reasons we have substituted the term "granulosis" for *pseudo-grasserie*.

Caterpillars of *Pieris brassicae* (Linn.) infected with the granulosis under consideration exhibit a whitish-yellow color on their ventral surface. At the time of death, the integument of the insect is rather fragile. When the skin is broken, a milky liquid flows out with an appearance similar to that characteristic of silkworm jaundice. Even earlier in the infection the blood is turbid and has a somewhat fluorescent quality. Microscopic examination of the blood reveals the presence of numerous minute granules or granulelike bodies measuring 0.2 to 0.3 micron in diameter. According to Paillot's (1926*d*) conception, the granules "multiply" in a manner similar to that of micrococci, and he likens the granules to the organism causing pleuropneumonia of cattle. The actuality of true biological fission in this case, however, needs confirmation.

The granular inclusions have been noticed only in the cytoplasm of the cells of the adipose tissue and in those of the hypodermis. In these infected cells, the nucleus becomes greatly hypertrophied. The chromatin takes on a "lacquered" appearance, and the nucleoli break up into bodies of very irregular form and size. These particles are forced out to the periphery of the nucleus. Toward the end of this morbid process the nucleus appears as an area without any definite structure and stains pale gray instead of dark purple with iron hematoxylin. The mitochondria of the cell move into the nuclear region.

The malady is a contagious one. Transmission is readily effected by either the ingestion or injection of infected material. There is no evidence that the disease is of any great importance in the destruction of caterpillars in nature.

Granuloses of *Euxoa segetum* Schiff.

Paillot considered the three granuloses (which he designated as "*pseudo-grasserie* 1, 2, and 3) of the cutworm *Euxoa segetum* Schiff. as three distinct infections. The relations between the three infections and their causative agents is still not entirely clear, and most of what we know about them is based on facts relating to their pathologies. The salient features of these may be summarized here.

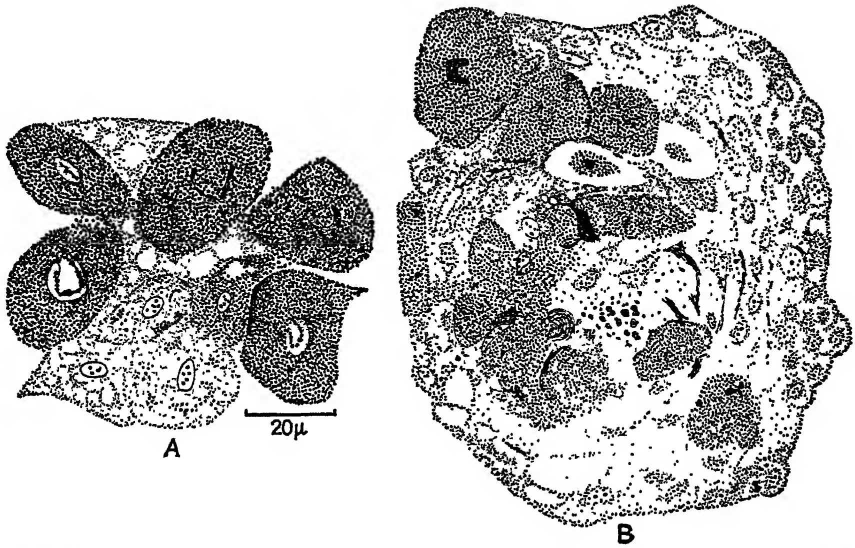


Fig. 160. Granulosis 1 of *Euxoa segetum* Schiff. *A*. Cells of adipose tissue showing various degrees of nuclear degeneration and the presence of the granular inclusions characteristic of the disease. *B*. Follicular nodule in the fat body of a diseased *Euxoa* larva, showing presence of granular inclusions, some of them no longer confined within the individual cell membranes. (*Adapted from Paillot, 1936.*)

Granulosis 1 of *Euxoa segetum* Schiff.

Granulosis 1, or as Paillot designated it, *pseudo-grasserie* 1, was observed in caterpillars of *Euxoa segetum* Schiff. collected in a suburb of Lyon, France, in 1934. It has not yet been reported outside of France. The disease is a mild one as far as the host population is concerned, and its incidence rarely exceeds 10 per cent in any one area.

The general symptoms of the disease are confined to a progressive weakening, sluggishness, and flaccidity of the host larva. The ventral region of the body has an abnormal white appearance because of the color of the diseased fat body.

When the body wall of an infected larva is opened, the abnormal porcelain-white appearance of the fat body is immediately noticeable. The normally slender, translucent bands of adipose tissue are obviously thicker and have lost their translucency. The blood is more or less turbid, depending upon the severity of the infection, and upon microscopic examination it is revealed to contain numerous small coccuslike granules exhibiting Brownian movement. Under dark-field illumination the granular mass is uniformly brilliant, and in contrast with the true bacteria the outlines of the granules are not clear. Microscopic examination of the adipose tissue reveals it to be the principal seat of the infection, its cells being filled with these same small granules. In size the granules measure about 0.2 to 0.3 micron in diameter. They stain faintly with fuchsin and with the Fontana-Tribondeau spirochete stain.

Histological sections of caterpillars stricken with granulosis 1 show a pathology limited largely to the adipose tissue. The pathological changes may appear varied throughout the fat tissue of any one insect, since the infection spreads from cell to cell but at a rate much slower than that characteristic of the polyhedral diseases. Also cells not yet infected may frequently be found at the periphery and in the anterior and posterior parts of the fat body. The granules first become apparent in the cytoplasm of the cells, where they increase in number until the entire cytoplasm becomes filled with them. In the meantime the chromatin of the nucleus loses its normal aspect and tends to form in a mass toward the center of the nucleus. At the end of the infectious process the residual chromatin stains black with hematoxylin and is often arranged in the form of an open crown. The mitochondria, normally rather elongated filaments, tend to fragment somewhat and to gather in masses in the interior of the cells, although later they may be forced out to the periphery.

The thickening of the bands of adipose tissue in the diseased cutworms is apparently the result of the proliferation of the fat cells, which are more numerous than in the case of healthy larvae. This proliferation of the adipose tissues is best seen in recently infected caterpillars but also may be apparent in those which are in an advanced stage of the disease. The uninfected cells that participate in the reaction assume the characteristics of young cells and multiply actively by mitosis.

Another type of cellular reaction noted by Paillot (1936), and probably related to the foregoing, is the formation of what Paillot calls "follicular nodules" at points in the fat body. These are analogous to the giant cells characteristic of tubercular infections in mammals. The center of one of these nodules is occupied by parasitized cells of the young type. Since there is no increased destruction of the parasitized cells within it, the nodule apparently is not a defense mechanism but represents a peculiar

type of cellular reaction, "une sorte de tourbillonnement cellulaire," the significance of which is not clear.

Granulosis 1 does not appear to be very contagious, since little success has been had in reproducing the disease experimentally either by the injection of infectious material into the body cavity or by the ingestion of such material by normal cutworms. Paillot assumes that in nature the

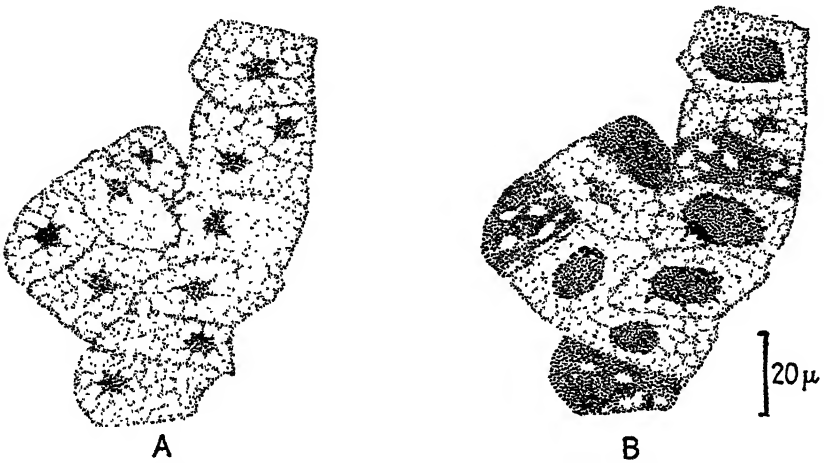


Fig. 161. Granulosis 2 of *Euxoa segetum* Schiff. *A*. Portion of adipose tissue from a normal *Euxoa* larva. (*Based on photograph by Paillot, 1936.*) *B*. Diagrammatic representation of same portion, showing pathological changes. Note hypertrophied and disintegrating nuclei and the characteristic small granular inclusions.

disease may be transmitted from one generation to the next by way of the egg.

Granulosis 2 of *Euxoa segetum* Schiff.

Although the granular elements of granulosis 2 (Paillot's *pseudo-grasserie* 2) of *Euxoa segetum* Schiff. are morphologically indistinguishable from those of granulosis 1, the histopathologies produced by the two viruses are fairly distinct. Paillot (1936) discovered this disease in the vicinity of Saint-Genis-Laval in France. The diseased cutworms have a flat-white aspect to the ventral part of their bodies, the enlarged fat body is porcelain-white, and the blood may be turbid or, in advanced cases, milky-white in appearance. The body wall usually appears more opaque than in larvae suffering from granulosis 1.

The virus of granulosis 2 has an affinity not only for adipose cells but also for the cells of the hypodermis and the tracheal matrix. The hypodermis is considerably thicker than that of normal caterpillars and presents numerous internal folds that are usually projected into the body cavity.

As the hypertrophy progresses the cells become filled with the minute granules associated with the disease. Most of the nuclei are destroyed and can be distinguished only faintly from the surrounding cytoplasmic layer. The chromatic and nucleolar substance appears in the form of siderophilic inclusions of variable size, thrown out to the periphery or distributed irregularly in the interior of the parasitic mass. The same nuclear alterations appear in the peritracheal layer, which appears con-

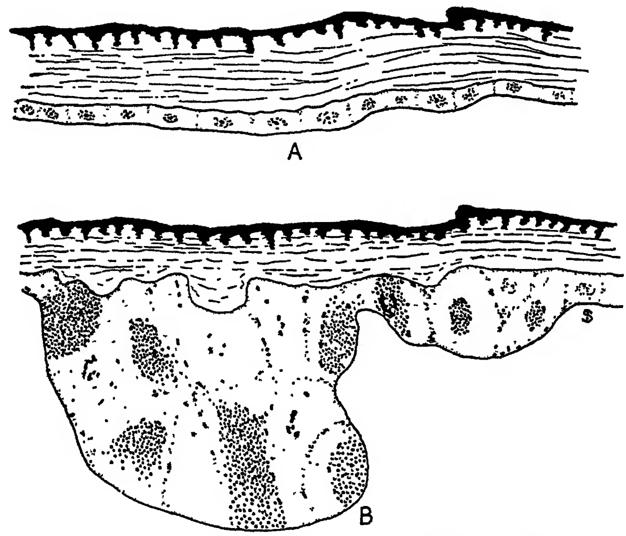


Fig. 162. Granulosis 2 of *Euxoa segetum* Schiff, showing pathology of the hypodermis. *A*. Transverse section of the integument of an uninfected *Euxoa* larva. *B*. Transverse section of the integument of an *Euxoa* larva infected with the virus of granulosis 2, showing the hypertrophied hypodermis and the granular inclusions characteristic of the disease. (*Redrawn and adapted from Paillot, 1936.*)

siderably more thickened than it does normally. Some nuclei become pycnotic.

The infected adipose cells hypertrophy and present an appearance similar to that we have described for granulosis 1. However, the process of cellular infection differs noticeably from that which occurs in granulosis 1. In granulosis 2, the granular elements appear and increase in the nucleus of the cell as well as in the cytoplasm. The nucleus of the infected cell loses its normal appearance early in the process with rather elongated strands, staining pale gray with hematoxylin and representing the parasitic granular mass, appearing in the interior of the nucleus. Bodies of various sizes, the largest being difficult to distinguish from the nucleoli, gather about the nuclear boundaries. The greater part of the nuclear space becomes occupied by the granules of the disease. Eventually the entire

cell fills with granules, the nuclear remnants forming crown-shaped or crescent-shaped masses that are dispersed here and there throughout the tissue. Sometimes the granules appear gathered into small irregular accumulations. In general the final histological appearance is similar to that of granulosis 1.

The follicular nodules mentioned in connection with granulosis 1 have not been observed in cases of granulosis 2. There is, however, marked proliferation of the adipose tissue. Not only does the fat body become more voluminous, but the number of cells increases markedly. Intermingled with the granule-filled cells may be seen others having the appearance of embryonic cells. Nuclei are in the process of active mitoses and multiplication. This cellular rejuvenation of the adipose tissue under the influence of a parasitic agent should be of interest to the medical scientist concerned with neoplasms. Other instances of this phenomenon are known in insects parasitized by other types of microbial agents. Several investigators have, for example, reported similar cellular proliferations in the case of microsporidian infections. From the standpoint of comparative pathology the subject would seem to merit further consideration and investigation.

Granulosis 2 is more contagious than is granulosis 1, but not nearly so much as are the polyhedroses. Infection can be experimentally effected by the direct inoculation of infectious material into the body cavity of the cutworm, but peroral infection succeeds only rarely.

Granulosis 3 of *Euxoa segetum* Schiff.

In 1937(*b*) Paillot discovered still a third "*pseudo-grasserie*" of *Euxoa segetum* Schiff. Like the other two, this disease was characterized by the presence of minute (0.2 to 0.3 micron) granules in the affected tissues which in this case, as with granulosis 2, are the adipose tissue, hypodermis, and tracheal epithelium. Unlike the larvae infected with the first two viruses, those infected with granulosis 3 may die without showing the usual white coloration through the body wall. Shortly after death, the larvae blacken and deliquesce. The disease is more malignant than are granulosis 1 and 2, its virulence approaching that of the granulosis of *Pieris brassicae* (Linn.).

The fat body of a larva stricken with granulosis 3 does not hypertrophy to so great an extent as in the other two infections. The nuclei of the adipose cells takes on a light brown tint, and their dimensions are considerably larger than normal. The nucleoli tend to group themselves into small masses, deeply stained by the hematoxylin. The granules gradually begin to appear in the nucleus, which hypertrophies and eventually becomes filled with the granules. The cytoplasm of the fat cells remains

vacuolated and is not replaced by granules as in the cases of granuloses 1 and 2. Toward the end of the morbid process the cell appears as a highly vacuolated mass at the center of which is the finely granular mass which occupies the place of the nucleus and which contains chromatin and nucleolar debris.

Cellular and tissue rejuvenation, as seen in the other granuloses, apparently does not occur in the case of granulosis 3. Follicular nodules, similar to those characteristic of granulosis 1, may be formed. Frequently there occurs an infiltration of the adipose tissue by the hemocytes, which more or less completely surround the diseased cells.

Granulosis 3 is very contagious. The cutworms are susceptible by the digestive route as well as by direct inoculation into the body cavity. At a temperature of about 10°C., the cellular lesions develop within 10 or 12 days after the ingestion of infectious material.

Granulosis of *Peridroma margaritosa* (Haw.)

Until 1947 no instance of a granulosis had been reported in insects outside of France. In that year an outbreak of disease occurred among variegated cutworms, *Peridroma margaritosa* (Haw.), being reared in an insectary in California. The mortality due to this disease was very high. Only a small percentage of the caterpillars ever reached pupation, and only about 50 per cent of the eggs produced by the moths that later emerged proved fertile. Preliminary examination of the diseased specimens revealed the malady to be of the same general type as the granuloses or *pseudo-grasseries* described by Paillot in the *Pieris* and *Euxoa* larvae (Steinhaus, 1947).[1] Microscopic examination of the cutworm revealed the presence of bacteria in the blood. The pathology of the fat tissues was characterized by the presence of large numbers of small granular or granulelike inclusions in the cytoplasm of the fat cells. The nuclei of the fat cells were usually hypertrophied and in a state of degeneration. The bacteria, considered to be secondary invaders, were gram-negative small rods that, for the most part, did not ferment lactose.

Healthy variegated cutworms can be infected by direct inoculation into the body cavity and apparently through the mouth by means of contaminated food. (There is also some evidence that the infecting agent may pass from one generation to the next in association with the egg.) After 2 or 3 days the infected insects begin to eat less food; they may remain

[1] In the original report (Steinhaus, 1947) the possibility was indicated that the granular inclusions may have some points of similarity with those incompletely described by Graham (1947) in the spruce budworm, *Choristoneura fumiferana* (Clem.) (*Archips*). Since then a direct microscopic comparison has been made, and it appears that no relation or close similarity exists between the two kinds of bodies.

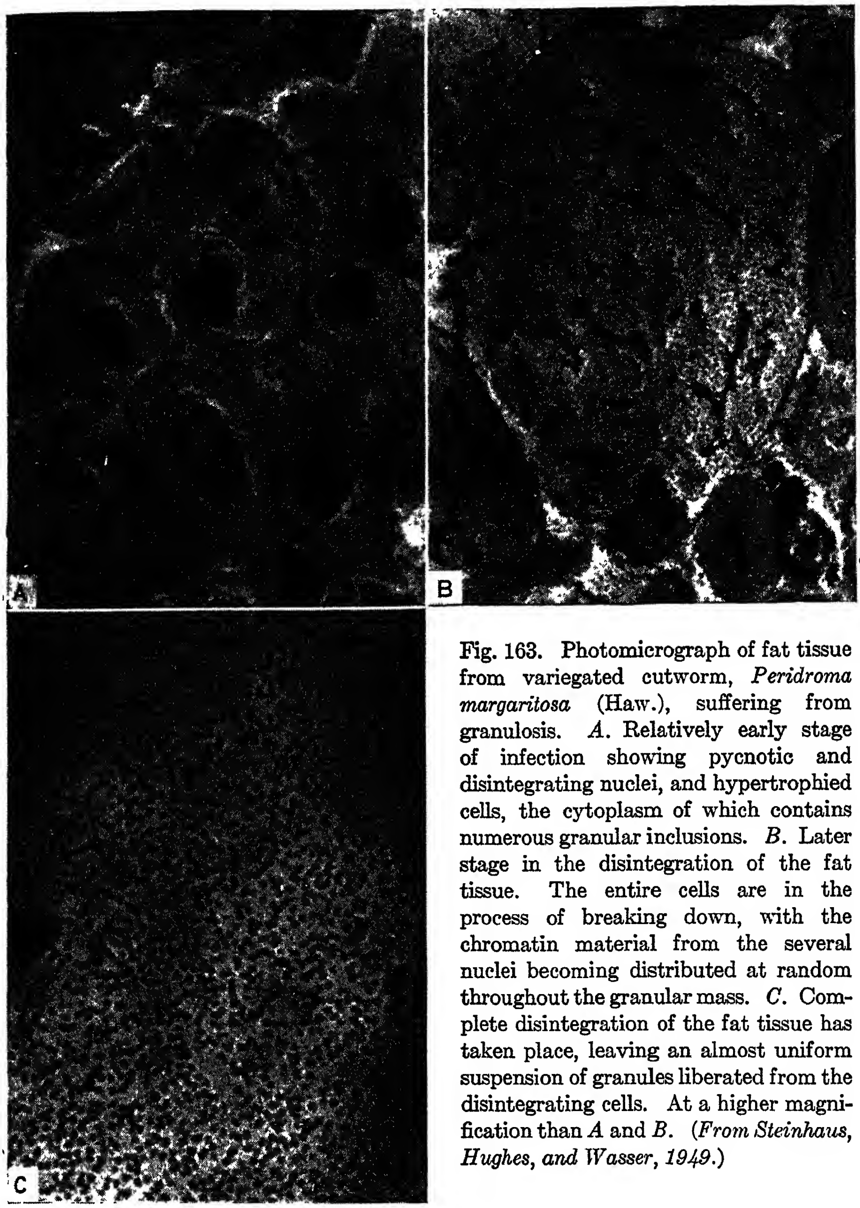


Fig. 163. Photomicrograph of fat tissue from variegated cutworm, *Peridroma margaritosa* (Haw.), suffering from granulosis. *A*. Relatively early stage of infection showing pycnotic and disintegrating nuclei, and hypertrophied cells, the cytoplasm of which contains numerous granular inclusions. *B*. Later stage in the disintegration of the fat tissue. The entire cells are in the process of breaking down, with the chromatin material from the several nuclei becoming distributed at random throughout the granular mass. *C*. Complete disintegration of the fat tissue has taken place, leaving an almost uniform suspension of granules liberated from the disintegrating cells. At a higher magnification than *A* and *B*. (*From Steinhaus, Hughes, and Wasser, 1949*.)

slightly smaller in size than normally developing insects and have a somewhat languid appearance, and, in the cases so far observed, they usually die before pupating. The fragility of the integument and the marked internal liquefaction of tissues, so characteristic of polyhedroses,

are generally absent. The larvae are flaccid, but the body wall remains relatively firm.

Upon dissecting a diseased larva one immediately notices an opaqueness of the fat tissue (normally having a clear appearance), which may be solidly white or in light infections may be only flecked with opaque white areas. Under a compound microscope these opaque areas may be observed to consist of nodules of hypertrophied fat cells filled with large numbers of minute granules, which may be seen with an electron microscope to have oval contours and to have an average size of about 0.4 to 0.5 micron long by 0.2 to 0.3 micron wide. Suspended in an ordinary wet mount, the infected cells break down rather rapidly, liberating the contained granules until eventually the entire preparation consists of millions of discrete granules together with some cellular debris. The granular inclusions are nearly elliptical, are not so refringent as are polyhedral bodies, possess a very slight tan or cream coloration when seen en masse, and are readily visible with an ordinary light microscope. When stained preparations are attempted, these bodies lose some of their granular aspect and appear as lightly stained amorphous particles, frequently coalesced. They do not appear to have the characteristic attributes of bacteria and are not cultivable on the usual bacteriological media. A few filtration experiments have shown the filtrates from Mandler filters of coarse porosity to be infectious but, so far, not those from Mandler filters of medium or fine porosity.

When placed in 0.04*M* sodium carbonate, the granules undergo dissolution. When such preparations are examined with the electron microscope, a single rod-shaped virus particle may be seen where the granule had been. Granules that have undergone only partial dissolution may still be seen to retain the virus particle or the cavity in which the virus particle was embedded (Fig. 164*B*). The rod-shaped virus itself has a size of roughly 40 by 340 millimicrons as measured with the electron microscope (Steinhaus, Hughes, and Wasser, 1949).

Sections of the diseased cutworms show a characteristic histopathology of the fat tissue. The nuclei of the fat cells appear either as considerably enlarged densely staining masses or as disintegrated particles of chromatin material scattered over an area that represented the orginally hypertrophied nucleus. The cytoplasm of these cells is packed with large numbers of the granular inclusion bodies. Sometimes the cell membranes are broken down so that the contents of several cells are enclosed in a single area the size of several cells.

Granulosis of *Cacoecia murinana* Hüb.

In the Black Forest region and other parts of Europe the fir-shoot roller, *Cacoecia murinana* Hüb., has been observed to suffer from a disease

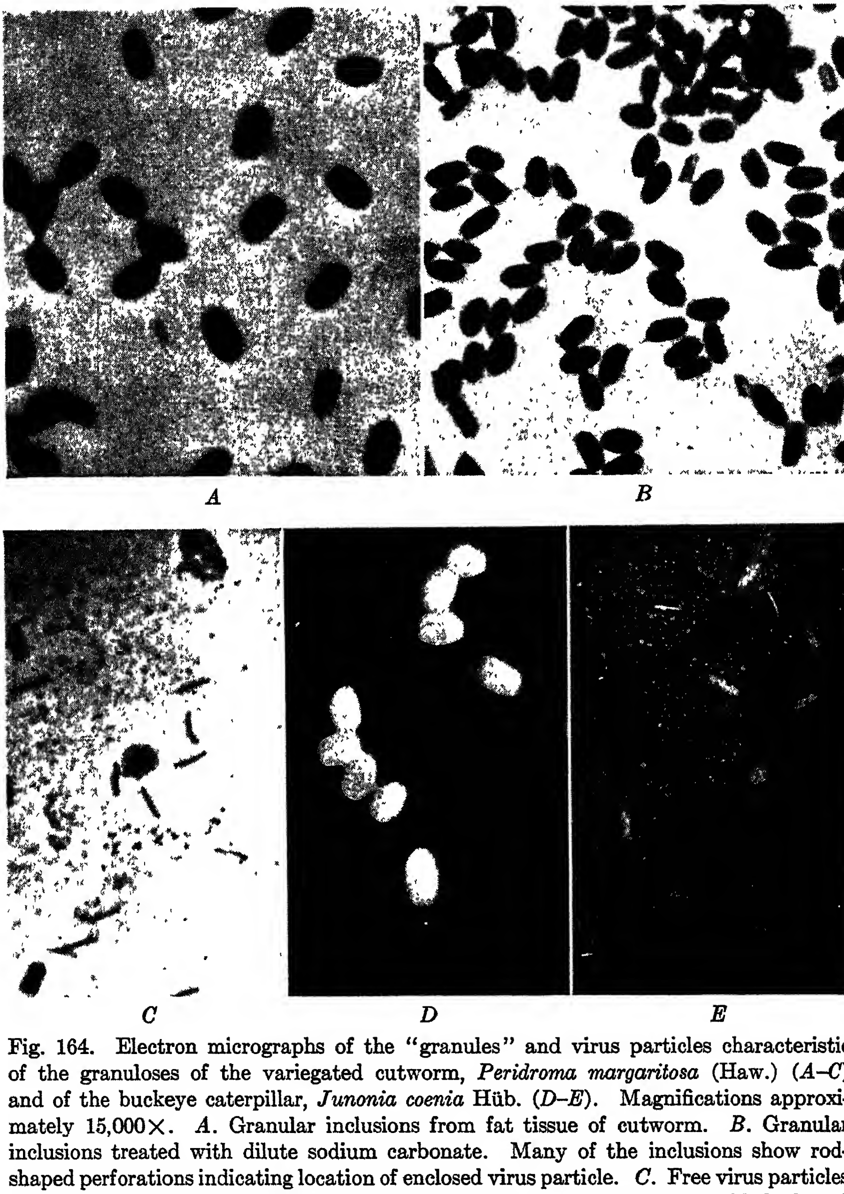


Fig. 164. Electron micrographs of the "granules" and virus particles characteristic of the granuloses of the variegated cutworm, *Peridroma margaritosa* (Haw.) (*A–C*) and of the buckeye caterpillar, *Junonia coenia* Hüb. (*D–E*). Magnifications approximately 15,000×. *A*. Granular inclusions from fat tissue of cutworm. *B*. Granular inclusions treated with dilute sodium carbonate. Many of the inclusions show rod-shaped perforations indicating location of enclosed virus particle. *C*. Free virus particles of the *Peridroma* granulosis. *D*. Granular inclusions (as seen in a gold-shadowed preparation) characteristic of *Junonia* granulosis. *E*. Almost completely free *Junonia* virus particles, gold-shadowed. The remains of the dissolved granules can be seen about the virus particles still partially covered with some of the granular material. The *Junonia* granulosis is apparently distinct from the *Peridroma* disease (see footnote page 501). (*Photographs by K. M. Hughes.*)

that was ascertained in 1948 to be one of the granulosis group. Examining material provided him by G. E. Bucher, Bergold (1948) saw the same type of granule that had been noted in earlier cases of this general type of disease. He then demonstrated without doubt that within each granule is located a single virus particle [which we have named *Bergoldia calypta* (see page 422)[1] (from Greek *kalyptos*, covered, hidden)] thereby proving the virus etiology of the disease.

Larvae infected with the virus show very few external symptoms of the disease until shortly before their death. At this time the normal yellowish-green insects become thickly swollen and are colored a pale greenish hue. When the integument of a diseased larva is punctured, a white milky fluid oozes out. Examination of this material with an ordinary light microscope reveals the presence of innumerable small granules occurring singly or clumped together in packs.

In the early stages of the disease, before any external symptoms can be recognized, the blood cells of an infected larva show in their cytoplasm one or more vibrating vesicular structures of variable size (from 1 to 50 microns). Later the thin membranes surrounding these structures break, and numerous, minute (less than 1 micron), hyaline granules are liberated.

Electron micrographs show these minute granules to be oval in shape and to have an approximate size of 230 by 360 millimicrons (Fig. 165). Sometimes several granules appear to be fused or clumped together into an elongated body. That the density of the granules is high is indicated by the fact that the electron beam does not penetrate them.

Unlike polyhedra, the granules do not dissolve in a solution of $0.008M$ sodium carbonate and $0.05M$ sodium chloride. In slightly higher concentrations ($0.02M$ sodium carbonate), however, rod-shaped cavities in each granule can be seen. In still stronger alkaline solutions ($0.03M$ and $0.04M$ sodium carbonate), the granules dissolve to the extent that the single virus particle enclosed in each one is released. The size of these rods is 50 by 262 millimicrons. Other properties include a Svedberg s_{20}

[1] In order to validate the generic name *Bergoldia* (see p. 422), it was necessary for the writer to designate the type species of the genus. *Bergoldia brassicae* (Paillot) (= *Borrelina brassicae* Paillot) was not adequately described by Paillot (1926*d*), and so little is known about it that under present circumstances it would be a very unsatisfactory type species for the genus. On the other hand, since the virus from *Cacoecia murinana* Hüb. was the first one obtained in a relatively free state and since a significant amount of critical information is available on it, the author feels that it would be the logical type species of the genus and that it could be more permanently associated with the genus than could *Bergoldia brassicae* (Paillot). In order, therefore, to make it available for designation as type species the author has chosen to name it—even though he has refrained, at this time, from doing the same with other unnamed species in the group.

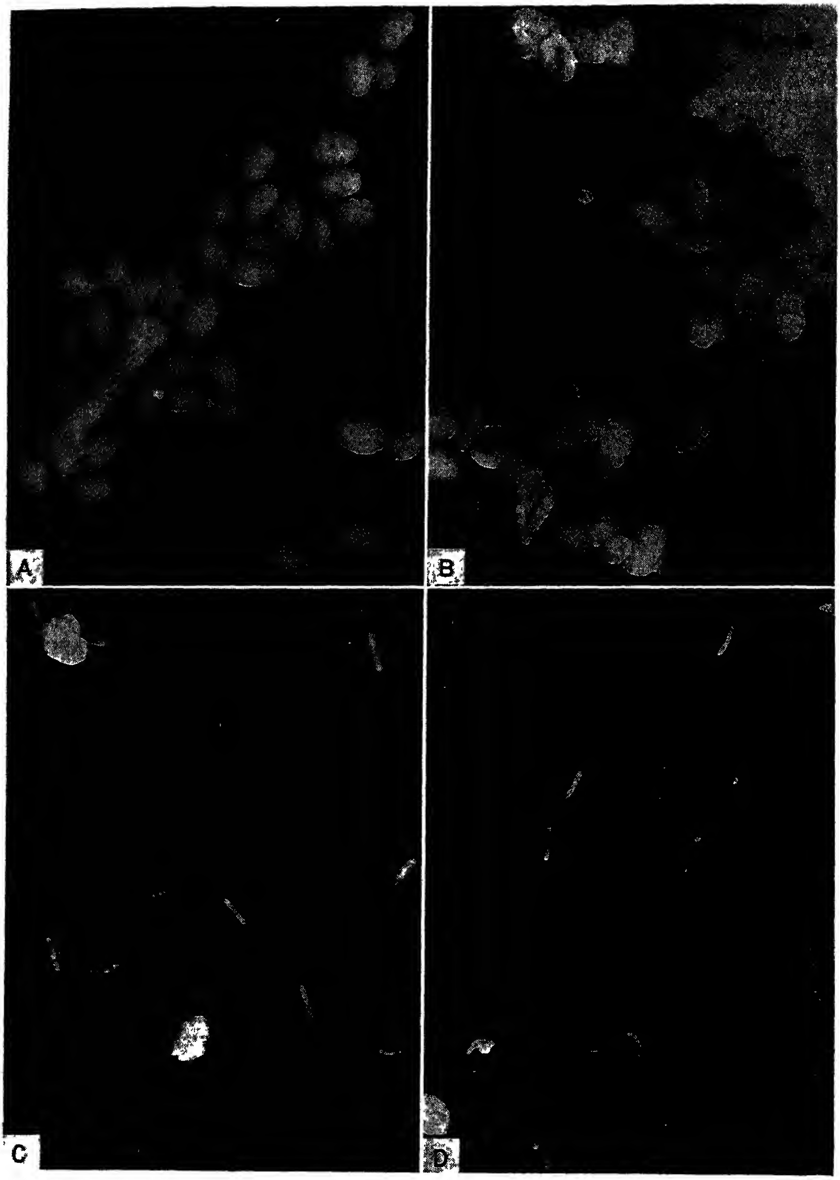


Fig. 165. Encapsulated virus of *Cacoecia murinana* Hüb. granulosis. Electron micrographs. *A*. The "virus capsules" or granular inclusions characteristic of the disease. *B*. The virus capsules from which the virus particles have slipped out after alkaline treatment, leaving rod-shaped perforations or cavities in the capsules. *C* and *D*. Free virus particles. Approximate magnification 25,000×. (*Courtesy of G. Bergold.*)

sedimentation constant of 1,324, a diffusion constant of 0.278×10^{-7}, a frictional ratio of 1.49, an axial ratio of 5.2, a particle weight of 460×10^6 when calculated from sedimentation and diffusion constants and of 435×10^6 when calculated from the length and diameter of the particle as seen on electron micrographs.

The material that surrounds the virus particle is protein in nature and is designated by Bergold as a "capsule." This "virus capsule," however, should not be confused with the polysaccharide capsules that surround such bacteria as the pneumococcus. The principal component of the material that surrounds the virus particle has a Svedberg s_{20} sedimentation constant of 11.8 and a molecular weight of about 300,000. The split components have a sedimentation constant of 3.45 and a molecular weight of about 60,000. These determinations were made with an analytical ultracentrifuge. The similarity in physical-chemical properties between these "capsules" and the polyhedra is evident even though one accumulates in the cytoplasm of the cell and the other in the nucleus. In contrast to the granulosis viruses, in which, as far as is known, only one virus particle is embedded in each capsule, the polyhedrosis viruses lie as numerous single rods or as numerous bundles of rods in the polyhedron.

The details of the pathogenesis and the histopathology of the disease remain to be worked out.

An Unidentified Infection

The larva of *Camptochironomus tentans* (Fabr.) is known to suffer from an infection characterized by the presence of minute particles which Weiser (1948) has referred to as "rickettsia-like organisms." According to Weiser, this parasite somewhat resembles the granules of Paillot's *pseudo-grasserie* 1 as seen in the cutworm *Euxoa segetum* (Schiff.).

The small granular bodies, 0.2 to 0.3 micron in diameter, attack the fat body and also circulate freely in the hemolymph. According to Weiser, the parasite attacks the fat body, fills the fat cells, destroys the cell membrane and multiplies in spherical balls (Fig. 166). The nuclei of the cells are not attacked. The tissue gradually disintegrates, liberating the tiny bodies into the hemolymph, which becomes milky-white.

A definition of the exact nature of this infection and its causative agent must await further study; it is possibly a granulosis.

VIRUS DISEASES NOT CHARACTERIZED BY THE PRESENCE OF CELLULAR INCLUSIONS

In comparison with the number of insects known to be susceptible to inclusion-producing viruses, the recorded number that are attacked by viruses which do not characteristically cause polyhedra or other inclusions

to be formed in the tissue cells is small. It is not unreasonable to suppose, however, that the number of noninclusion virus diseases that actually exist is considerably larger than is indicated at present. Nevertheless such diseases apparently are not so numerous or obvious in nature as are the spectacular, easily recognizable polyhedroses; otherwise there undoubtedly would have been more such instances reported than has been

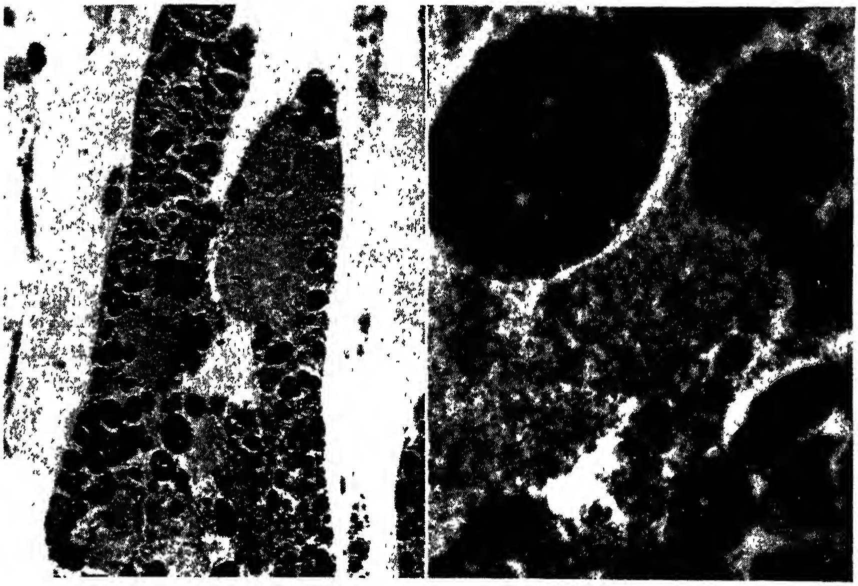


Fig. 166. "Rickettsia-like" infection of *Camptochironomus tentans* (Fabr.). *A*. Fat body of infected larva as viewed with the low power of a microscope. *B*. Infected fat tissue at a higher magnification, showing the minute parasite and the disintegrating tissue. (*Courtesy of J. Weiser.*)

the case. A large proportion of the virus infections of higher animals and plants is not characterized by the presence of inclusion bodies, and one might justifiably assume that a considerable number of similar infections prevails among the Hexopoda.

Principally for the sake of convenience, the word "nonpolyhedral" has sometimes been applied to this group of viruses. Several objections might be raised to the use of this term. As we have seen, polyhedra are not the only kinds of inclusion bodies capable of arising in and being contained by diseased cells. Correctly speaking, therefore, the term "nonpolyhedral" does not exclude those diseases which are characterized by inclusions other than polyhedra—the inclusion disease of *Pieris brassicae* (Linn.), for example—and for this reason is inappropriate. The diseases

with which we shall be concerned in the following few pages are diseases caused by ultramicroscopic viruses, but they are diseases in which no cellular inclusions of any kind are visible by the ordinary light microscope.

Sacbrood of the Honeybee

Published accounts of what was probably the disease of the honeybee (*Apis mellifera* Linn.) we now know as "sacbrood," appeared as early as 1857. In that year Langstroth referred to two types of "foul-brood," one called the "dry" type, the other known as the "moist" or "fetid" type. The dry type may have been sacbrood. Following this, reports by such men as Doolittle, Jones, Simmins, Cook, and others all made mention of a condition affecting the brood of honeybees that might very well have been sacbrood. Early reports of Burri and that of Kürsteiner in Switzerland were probably also concerned with this malady. In 1902 G. F. White, in New York State, became interested in the disease and proceeded to make what is still perhaps the most thorough investigation into its nature and characteristics. It was he (1913) who gave to the disorder the name "sacbrood." In 1917 White published the results of his investigation, and this report must of necessity (for the lack of recent comprehensive research) serve as the source of most of the information for any present discussion of sacbrood. In the paragraphs to follow we have made liberal use of White's account.

Symptoms and Gross Pathology. In considering the symptoms of sacbrood and the gross pathological changes gradually brought about in the honeybee by the infection, one should study the colony as a whole as well as the individual bee. Not only is the beekeeper himself likely to consider the effect of disease on his colony as a unit, but changes in the colony's over-all aspect are frequently as significant as are changes in the appearance and activity of an individual insect. Of the colony, only the brood suffer the effects of sacbrood; the adult bee does not appear to be susceptible.

Among the first symptoms of the colony noticed by the beekeeper are the presence of dead brood and the irregularity of the brood nest. If the disease is severe, the colony is noticeably weakened, and the loss of strength may become serious. Frequently, however, the strength of the colony is not appreciably diminished. Brood dying of the disease does so almost invariably in capped cells but before the pupal stage is reached. Only on rare occasions are pupae found that have been killed by sacbrood. When dead larvae are found in uncapped cells, it usually represents cases in which the adult bees have removed the caps from the cells—as if they were trying to determine what was wrong with the immature bee

inside. Sometimes, instead of removing the entire cap, the bees puncture a hole or two in this covering.

Inside the cell the dead larva is usually found lying extended lengthwise with its dorsal side on the floor of the cell. If recently dead, the insect is slightly yellow in color but in a few days it becomes brown, and as the process of decay continues it eventually appears almost black. Sometimes during the process of decay, the larva presents a grayish appearance. The

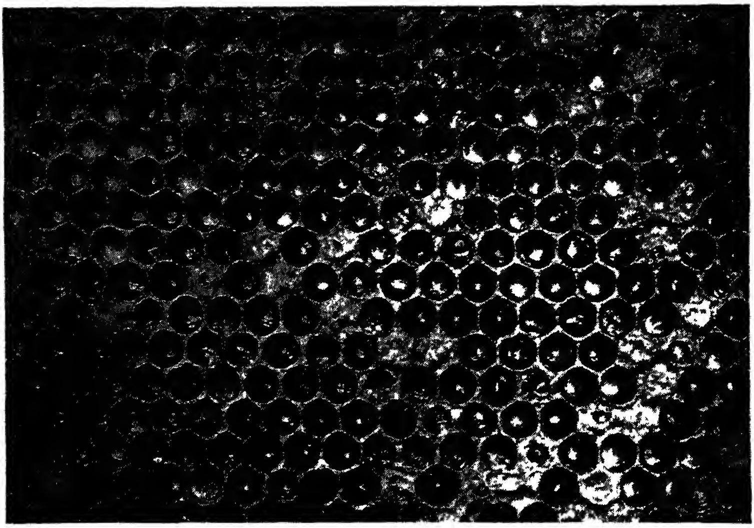


Fig. 167. Brood comb showing numerous honeybee larvae dead of sacbrood. (*Courtesy of C. E. Burnside.*)

body wall or integument of the dead insect toughens, permitting the saclike remains to be easily removed intact from the cell. The contents of the larval remains, during a certain period of its decomposition, are watery and granular in appearance. Gradually the watery content is evaporated and the insect becomes wrinkled and distorted in its form, until finally it drys down to a "scale," which is not adherent to the cell wall. Brood combs affected with sacbrood have no appreciable odor; later, after the process of decay is well under way, the crushed insects do have a disagreeable odor.

Since the time when larvae succumb to sacbrood is usually during a period when they are motionless, it is not always easy to determine the exact time at which they die. Usually they may be considered moribund or dead when they begin to show some change in color—usually from a bluish-white to yellowish—or when they lose their normal turgidity and become flaccid. The changes and differences in appearance that

ensue from this time on vary from day to day. For this reason White, in describing larvae dead from sacbrood, divided the gradual and continual changes into five more or less arbitrary stages.

First Stage. A larva showing the first symptoms of the disease has a slightly yellowish appearance that may deepen slightly later on during the stage. The lateral margins of the anterior third of the larva and the extreme cephalic end usually assume a transparent appearance. The position and surface markings are essentially the same as those of a normal larva. Sometimes the extreme anterior end settles somewhat and drops away from the roof of the cell a little. The transverse ridges and furrows of the middle and posterior thirds remain well marked, and under slight magnification, the transverse tracheae may be distinctly seen. The lateral and posterior margins are still deeply notched and often appear transparent, which is due to a watery-looking fluid beneath the cuticular portion of the body wall. The cuticle is less easily broken at this time than in the healthy insect. When the integument is ruptured the fluid tissue mass, which is less milky in appearance than that from a normal larva, flows out; its granular appearance, due chiefly to the presence of fat cells, is noticeable but not so marked as it becomes in later stages of decay. The dead larvae are particularly infectious at this stage. This fact is particularly important, because it is during this stage that the dead larva is frequently removed piecemeal from the cell by the workers which thus aid in the dissemination of the infectious agent.

Second Stage. The yellowish color of the first stage has become a brownish tint, although in the case of some larvae traces of yellow remain. The shade of brown is usually deeper in the anterior third than in the other two-thirds. The apex is farther from the roof of the cell. In the posterior two-thirds of the larva, the segmental ridges and furrows are less pronounced; the lateral margins are still deeply notched. The cuticular sac is now more readily observed and less easily broken. The subcuticular fluid at the lateral and posterior margins has increased in quantity. The contents consist of a brownish granular mass of disintegrated tissue cells suspended in a watery fluid. The remains of the larva at this stage are still infectious in some instances but less so than in the first stage.

Third Stage. The dead larva of this stage is brown in color, the anterior third being a deeper brown than that of the other two-thirds. In a general way the larva still retains the form and marking of a normal larva, although the normal turgidity is gone. The cuticular sac is very tough, permitting the removal of the larva from the cell with considerable ease and with little danger of its being torn. The granular content of the sac is still brownish in color and is suspended in a small quantity of clear watery fluid. White (1917) found that, when the larval remains in this stage of

decay are crushed and fed in sirup to healthy colonies, no sacbrood is produced, indicating that the dead larva at this stage is not infectious and that the virus is probably dead or inactive.

Fourth Stage. Marked evidence of drying characterizes this stage. The brown color of the larval remains has deepened; the anterior third

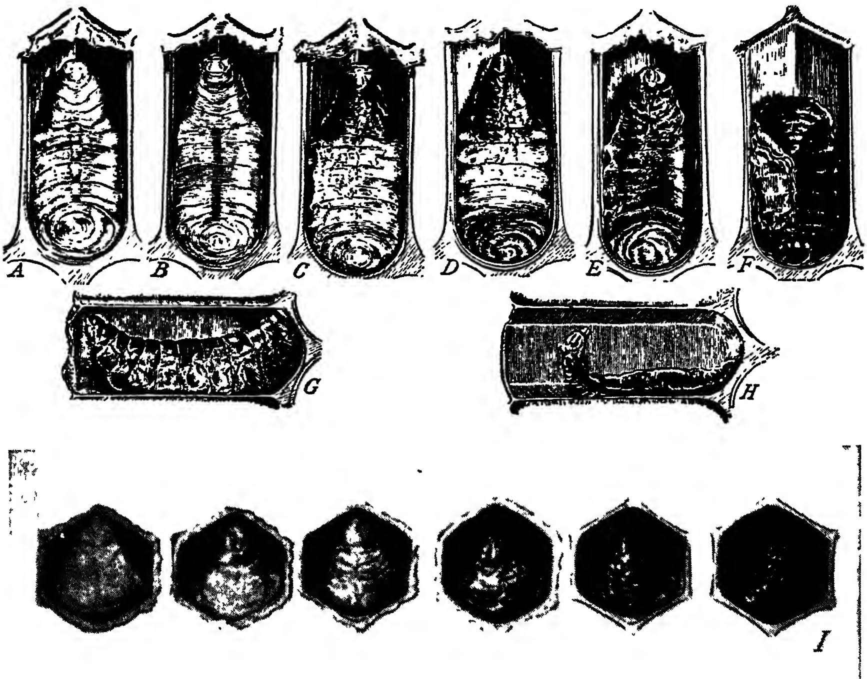


Fig. 168. Larvae of the honeybee, *Apis mellifera* Linn., showing certain of the symptoms of sacbrood. *A*. Ventral view of a healthy larva at the age when death from sacbrood usually occurs. *B–F*. Stages in the decay and drying of larvae dead of sacbrood, ventral views. *G*. Lateral view of larva recently dead of sacbrood. *H*. Lateral view of scale. *I*. End views of *A–F* above. (*From Burnside and Sturtevant, 1936.*)

may be such a dark brown that it appears almost black. As a result of drying, the apex of this conelike third is often nearer the roof of the cell than in the preceding stage. The surface markings are becoming less distinct, and the surface is becoming wrinkled because of the effect of drying. The subcuticular fluid is no longer present. The decaying tissue mass still appears granular, and the contents are pastelike in consistency. The larval remains do not appear to be infectious.

Fifth Stage. By the time this last stage is reached the dead larva has lost all its moisture through evaporation, and the dry mummylike remains are known as the "scale." They are noninfectious and may easily be

removed intact from the cell. The anterior third is retracted from the mouth of the cell, with the apex drawn still deeper into the cell and raised toward the roof of the cell. This third is very dark brown in color, almost black, and is greatly wrinkled. The dorsal side of the middle and posterior thirds is shaped to conform to the floor of the cell, being in general convex, with a surface that is smooth and polished. The margin is thin and wavy. The anterior third and the lateral sides of the middle and posterior thirds being turned upward, the ventral surface being concave, and the dorsal side being convex, the scale usually presents a boatlike appearance and could be styled "gondola-shaped." Beekeepers sometimes refer to it as having the form of a "Chinaman's shoe." When completely dry, the scale is brittle and may easily be ground to powder.

Histopathology. A few histopathological facts pertaining to sacbrood in the honeybee have been ascertained, but undoubtedly there remains much to be determined; White's (1917) observations on the histopathology of the disease are in need of being carried further. In the light of some of Paillot's work on the virus diseases of insects, it would be of interest, for example, to know what changes may take place in the mitochondrial make-up of the affected cell or what alterations may occur in the nucleus and cytoplasm of such a cell. Be this as it may, however, White has provided us with some interesting and valuable histopathological data. For instance, one of the chief diagnostic signs of sacbrood, the subcuticular waterlike fluid and its granular contents, can be better understood if the histology of the dead larva is considered.

A section through the body of a larva dead of sacbrood shows that a large part of the insect consists of fat tissue. The cells making up the fat tissue are comparatively large and are irregular in outline, having irregular-shaped nuclei. More or less spherical black bodies of varying size are contained within the fat cells. Glaser (1928) believes that these bodies may represent the so-called "protein bodies" found within the fat cells of insects prior to and during metamorphosis. At any rate, these fat cells are the chief cause for the granular appearance of the contents of the larva dead of sacbrood. The granular aspect is enhanced, however, by the presence of other cellular elements such as oenocytes, and other tissue cells.

A cross section reveals that between the molt skin, which is at a considerable distance from the hypodermis, and another cuticula lying near the hypodermis is an intercuticular space filled with a watery-looking fluid. That the fluid is not pure water is indicated by the fact that it coagulates during the preparation of histological sections. It consists chiefly of the blood of the larva, or fluids derived from the blood, and is

probably augmented by other liquids of the insect and by the liquefaction of some of the tissues. The molt skin is normally shed about 3 days after the cell containing the larva is capped. It is usually this skin which, for the most part, constitutes the sac that encloses the decaying larval mass in sacbrood.

The Virus of Sacbrood. As has already been mentioned, the remains of larvae recently dead of sacbrood are very infectious when fed to bees in sirup. A single larva contains enough infectious material to kill at least 3,000 healthy larvae in 1 week. On the other hand, dead larvae that have remained in the brood comb more than 1 month appear to be noninfectious. Using recently dead larvae, White (1913) prepared suspensions that retained their infectivity after having been passed through Berkefeld as well as through Pasteur-Chamberlain filters. No visible microorganisms of any kind could be seen in the filtrates, and none could be cultured on artificial media. Accordingly, White concluded that he was dealing with a filterable virus, now known by the name *Morator aetatulae* Holmes. This was the first proved instance of a filterable virus, infecting insects, not accompanied by the production of polyhedral bodies in the tissues of the diseased insect.

The virus of sacbrood is frequently aided in bringing about its ill effects by certain predisposing causes, some of them frequently referred to by beekeepers as the "primary cause" of the malady. In this connection the possible role of such factors as the age, sex, and race of the bees, and the climatic conditions, season, and food are interesting to consider. The age or another way of putting it, the stage of development of the bee, is quite important with regard to the degree of susceptibility shown by the insect. The greatest susceptibility to sacbrood is exhibited by the honeybee larva, which almost invariably dies of the disease after the cell is capped and usually during the 2-day period immediately preceding the time for the change to the pupal stage. The pupae are rarely affected, and when they are it is usually just after transformation from the larval stage. Adults are not directly susceptible to the disease. Worker, drone, and queen larvae all appear to be susceptible. No particular race of bees is known to be entirely resistant to sacbrood; Italians seem to be slightly more resistant than do blacks. Climate does not appear to have a direct effect upon the disease, since it has been reported from widely separated points in the United States and from several different countries (England, Germany, Switzerland, Denmark, Australia, and Canada). With regard to seasons, it may be said that sacbrood appears most often and in greatest severity during the spring of the year. It may, however, appear during any season of the year in which brood is being reared. The occurrence of

the disease does not appear to depend upon food of any restricted character, nor does the quantity of food available to the colony appear to be important in this regard.

Many properties of the virus of sacbrood, particularly its ability to withstand various environmental conditions, were determined by White (1917) using bees from experimental colonies. In brief, he found that the virus of sacbrood had qualities of resistance similar to those of most other viruses and of many bacteria. Suspended in water and heated to 59°C. for 10 minutes, the virus was rendered inactive. When suspended in honey, it could be destroyed by heating for 10 minutes at approximately 70°C. It withstood drying at room temperature for approximately 3 weeks. Dried virus exposed to the direct rays of the sun was destroyed in from 4 to 6 hours; when suspended in honey it was destroyed in from 5 to 6 hours. When suspended in honey and shielded from the direct sunlight, the virus remained virulent for slightly less than 1 month at room temperature during the summer. In the presence of fermentative processes taking place in a 10 per cent cane-sugar solution at room temperature, the virus was destroyed in about 5 days—the same period of survival as in a 20 per cent honey solution at outdoor temperatures. In the presence of putrefactive processes the virus remained virulent for approximately 10 days. The virus will resist 0.5, 1.0, and 2.0 per cent phenol for more than 3 weeks.

Transmission. The virus of sacbrood is transmitted from diseased to healthy brood within a colony by way of the intestinal tract through the ingestion of contaminated food. Experimentally it has been shown that the disease may be produced by adding the virus directly to their food such as mixing it with sirup and feeding it to the colony.

The tendency of adult bees to remove diseased or dead larvae from the cells, usually piece by piece, appears to be a means of disseminating the virus. If the removed fragments were fed to healthy young larvae within a week, sacbrood would very likely be produced. This may constitute one of the ways in which the disease is transmitted within the hive. It is not known, however, if feeding these bits of diseased tissue to young larvae is a frequent occurrence. If such were the case, it would seem that the disease would increase more rapidly than it usually does.

It is possible that other modes of transmission exist, but so far definite proof of such is lacking. Infectious material may reach the water supply of the bees, and some of the virus may thus be returned to the hive and reach healthy young larvae. The probability that the virus will be carried to flowers visited by bees and then picked up and returned by other bees appears to be remote. Stray bees drifting from infected colonies to healthy ones might be considered as possible transmitters, but there is no evidence

that the disease is spread to any great extent in this way. The robbing of weakened colonies by the bees of neighboring hives is a probable means by which the virus is carried from a diseased to a healthy hive.

The possibility that the virus will be transmitted by the hands of the operator, by the tools used about the apiary, or by the wind does not appear to be great from the standpoint of practical apiculture.

Treatment and Control. According to White (1917), the tendency in a colony affected with sacbrood is to recover from the disease. Colonies that during the spring months show signs of the disease, by midsummer or earlier may contain no diseased brood. Colonies may die out as a consequence of the disease, but the percentage that does is small. The usual harm brought on by sacbrood is the weakening of the colony to such an extent that the profits on it for the season are reduced or entirely eliminated. The disease may also cause the colony to be in a weakened condition on the approach of winter.

Since sacbrood usually is not a very serious disease, special treatment is not always necessary. In any case, it is never necessary to destroy the combs from sacbrood colonies on account of the disease. When convenient, it is well to store the combs for 1 or 2 months before they are used again. Chemical disinfection of the combs usually is not a dependable procedure. Since sacbrood cannot occur in the absence of the causative virus, anything that destroys the virus or assists in its dissipation will aid in controlling the disease.

The inclusion of sulfa drugs in the food does not appear to be of very much value in the treatment of sacbrood.

Paralysis of the Honeybee

The literature on the diseases of the honeybee, *Apis mellifera* Linn., contains numerous references to "paralysis" as it occurs in the adult stage of this insect. Sometimes the term "paralysis" is inadvisedly used in referring to some of the rather well-known afflictions of the honeybee, and at other times it is used to designate certain ill-defined disorders that are noticed from time to time by beekeepers in the United States and in Europe. Probably several disorders of the adult honeybee have inadvertently been considered as one disease and grouped under the heading of "paralysis." In recent years at least one of these conditions has been fairly well separated out and identified as a more or less distinct entity. We refer to the paralysis of honeybees that Burnside has described as being caused by a filterable virus. In 1933 this worker made one of the first scientific reports dealing with the nature and cause of this condition, which he showed to be an infectious disease. Butler (1943) confirmed this demonstration of its infectiousness before Burnside proved conclusively,

in 1945, that the etiological agent was a filterable virus. The present account is based upon the findings of Burnside as published in his two reports.

Symptoms. Bees affected with virus-caused paralysis become somewhat lethargic and do not respond readily to stimuli. They appear weak and are reluctant to fly. Recently infected bees hum feebly when disturbed but soon become quiet again. Later they appear stupified and fan their wings feebly. In the colony they can be recognized even in the earlier stages of the disease by the fact that other bees tug and pull at them excitedly. The ailing bees put up no defense, but sometimes they offer food or attempt to crawl away from their tormentors. Eventually they are driven out of the hive, or else they crawl onto the top bars or into a semi-quiet corner of the hive. Sick bees may either retain their hair or become partly or nearly hairless before death occurs. The old hairless bees usually appear to have shining, swollen, greasy, or translucent abdomens. According to Burnside, the most characteristic symptoms are decided trembling of the body and wings, particularly when accompanied by weakness, sprawled legs and wings, hairlessness, and dark, greasy-looking abdomens. Affected bees may either die quickly or linger in a weak condition for several days before death. From the standpoint of the colony, the disease may be very severe and destructive in its effects, or it may be mild or transient in character, with only a few bees being affected.

The Virus. Burnside has shown that the etiological agent of the form of paralysis under discussion is an ultramicroscopic filterable virus. A centrifuged suspension of triturated infected bees may be passed through a Chamberlain-Pasteur F filter or through a Coors porcelain bacteria-withholding filter one, two, or three times, and retain its infectivity for healthy bees. When such filtrates were sprayed into cages of healthy bees, 25 to 100 per cent of the bees developed typical symptoms after 8 to 14 days. After symptoms appeared, the death rate rose sharply and continued high for another 9 to 14 days.

Heating the infectious material at 93°C. for 30 minutes destroys its virulence.

Some strains of virus appear to have less virulence than others. In such instances there may be a considerable delay before symptoms appear, and these may be less pronounced. Sometimes death of the bees does not occur until 30 to 40 days after exposure to the virus.

Treatment. According to Eckert (1948), fairly effective treatment for a diseased colony is to dust flowers of sulfur over the entrance of the hive and the ground in front of the hive and over the bees and top bars of the hive bodies. Two dustings are sometimes needed, but the sick bees are reduced in number until after about 10 days little evidence of the disease

remains. It appears probable that the sulfur affects the sick bees and not the healthy bees, thus eliminating the reservoir of infection.

Virus-caused Dysenteries in the Silkworm

One of the most common descriptive terms in the early literature of insect pathology is the word *flacherie,* or its Italian and English equivalents *flaccidezza* and flachery. It was used to describe the flaccid condition seen in silkworms, *Bombyx mori* (Linn.), suffering from dysenteric conditions presumably brought on by the rapid development of certain bacteria in the alimentary tracts of the insects. Since these dysenteries usually caused the afflicted larvae to appear flabby, feeble, weak, withered, or loose-hanging, any term referring to this flaccidity was an apt one and became widely used to describe many different diseases. In fact, throughout much of the earlier writing the word "flacherie" was used indiscriminately for various infections of differing etiology in different species of insects, as well as for a general term implying a diseased condition accompanied by diarrhea. Today we know somewhat more about these conditions, and the indefinite word "flacherie" is no longer a satisfactory one when it is applied to different diseases of varying etiology. The terms "dysentery," "diarrhea," "septicemia," and the like are much more explicit and meaningful. Since the term "flacherie" has been used most frequently in referring to one of the diseases of the silkworm in which the bacterium *Bacillus bombycis* auctt. is involved, it seems best to avoid confusion by limiting its use to this particular condition, which we shall discuss shortly.

Historical Aspects. The two conditions that concern us here are caused by a virus that is followed in one case by a certain streptococcus and in the other case by a sporeforming bacillus. To understand these infections properly, however, as well as much of the literature concerning them, it is essential to have some idea as to the history of the investigations pertaining to their etiology.

As might be expected, early authors did not make a clear distinction between the various intestinal diseases of the silkworm, and it is therefore frequently difficult to ascertain just which affliction they were writing about. According to Paillot (1930*b*), who has briefly summarized the historical aspects of these diseases, one of the first to publish a fairly accurate description of the intestinal diseases of the silkworm was the abbot Boissier des Sauvages, in 1763. Like the earlier accounts, however, his did not distinguish the several different diseases that today are recognized as distinct entities. It is possible that the disease of "*passis,*" which he describes, is the infection now designated as "true flacherie," previously designated in France as "*morts-flats.*" In 1808 Nysten described the symptoms of a disease known as "*clairette*" or "*luzette,*" which now

goes under the name of *gattine*, or as designated by some because of the transparency of the head of the infected insects, "the disease of the clear heads." Thus the two diseases with which we are here concerned, true flacherie and gattine, may be said to have been more or less definitely recognized by the early part of the nineteenth century. In spite of this, the situation was kept somewhat confused by the not very penetrating works of some of the authors (*e.g.*, Cornalia, Lambruschini, Maestri, and de Quatrefages) writing on this subject about the middle of the last century. Some of these workers confused the dysenteric diseases with the protozoan infection pebrine, and with the fungous infection muscardine. Others attribute them solely to the effects of abnormal temperatures, excessive humidity, and improper ventilation.

It was Pasteur (1870) who definitely separated the dysenteries (flacherie) from pebrine as well as from muscardine and grasserie (jaundice) and who attributed them to microbial or infectious causes. This illustrious French scientist described flacherie as being characterized by the presence of a large number of certain kinds of bacteria in the intestinal tract of the affected silkworm where, because of the extremely rapid multiplication of these bacteria, the digestive functions of the gut were adversely altered, giving rise to the symptoms typifying the disease. Two species of bacteria seemed to be particularly important in this regard. One was a coccus arranged more or less in chains, which Pasteur called "*ferment en chapelets de grains*" and which today bears the name *Streptococcus bombycis* Zopf. The other was a sporeforming bacillus, Pasteur's "*vibrion à noyau*," now known as *Bacillus bombycis* auctt. The contributing effects of certain environmental conditions, such as adverse temperature and humidity, were also recognized. The contagiousness of the disease, however, was definitely established.

Although Pasteur's contributions were of great significance in determining the true nature of flacherie, they were by no means universally accepted. Verson, for example, did not believe there was any connection between the disease and the rapidly multiplying bacteria of the gut, except in larvae already in a state of disease. The true cause of the malady, according to Verson, was the obstruction of the Malpighian tubes by uric acid crystals, which resulted in certain disturbances of nutrition and assimilation and led to a decomposition of the blood. The germ theory prevailed, however, and the parasitic nature of the disease was affirmed by such workers as Conte, Cuboni, and Garbini; Macchiati; and Lo Monaco and Giorgi. Certain Japanese authors (*e.g.*, Sawamura) ascribed the cause to no particular bacterial species but rather to any of the numerous species that happen to be present on the mulberry leaves when these were fed to susceptible silkworms. On the other hand, Ishiwata isolated a spore-

forming bacterium ("*Bacillus sotto*") which he believed was very important in the etiology of flacherie. This assumption was later discounted by Paillot.

Thus we see that most of the early observations on the etiology of flacherie were not altogether reliable. The whole gamut of causative factors was run—from the miasmic and amicrobial causes to not only one but many kinds of bacteria. At this point in perusing the early literature one has the feeling that something very significant was being overlooked in all these investigations and that somehow the missing piece in the jigsaw puzzle had not yet been discovered.

Modern Concepts. Undoubtedly the last word concerning the rather complex etiology of flacherie is yet to be written. Some definite progress has been made since the time of Pasteur, however, and our present concept of the disease is probably nearer the truth than it was at that time. Even today, though, there is no unanimity of opinion as to just what constitutes flacherie and as to the exact nature of the etiological factors. The work of Paillot (1930*a*,*b*, 1941*a*) on this problem, however, has given the whole picture new color, and we cannot but accept his theories at their face value until they are definitely proved or definitely discounted. In any case Paillot's explanation of the etiology of flacherie seems considerably more convincing than is that of any of his predecessors.

According to Paillot, the term "flacherie" is a general one; and, as we have stated, it has been used in the past to include all sorts of dysenteric conditions—microbial and amicrobial—which have been observed in the silkworm. Of these, the two most important infectious dysenteries are gattine and true flacherie or "the flacherie of Pasteur." The exciting cause of both these conditions is an ultravirus to which bacteria are important secondary invaders. In the case of gattine, *Streptococcus bombycis* is the secondary invader; in true flacherie, or "the flacherie of Pasteur," the secondary organism is *Bacillus bombycis*. In a strict sense, therefore, neither *Streptococcus bombycis* nor *Bacillus bombycis* is the cause of the disease, which is actually initiated by an ultramicroscopic virus. For this reason, the diseases with which we are here concerned may be considered as true virus diseases and may properly be included in this chapter.

Although the inciting cause of both gattine and true flacherie is probably the same virus, the two syndromes are so distinctly different that the only practical way of discussing them is to consider them as more or less distinct entities.

Gattine

In Italy the disease we are about to consider is referred to as *macilenza* or as *gattina*. The latter is a form of *gattino* (kitten) and may refer to vomit

and to spoilage. The French adopted a modification of this Italian name in the form of *gattine*, which has also been accepted in English literature as the name of the disease. In older literature, the names *clairette* and *luzette* apparently refer to the same disease. These terms were probably meant to convey the same meaning as a phrase used by European writers, "the disease of the clear heads." This designation arises from the fact that the anterior or cephalic end of the infected silkworms frequently becomes swollen and practically translucent.

In addition to the "clear heads," other external symptoms of gattine include the disinclination of the affected silkworms to eat and the ejection from their mouths of a clear ropy liquid. Predisposing causes, such as temperature, humidity, and general state of health, do not appear to play so important a role in the production of epizootics of the disease as one might suppose. Regardless of the environmental conditions of the silkworm, if an infective dosage of the causative virus is ingested, the disease will result. Apparently infection may also occur by way of the egg of the insect, since there is strong evidence that the virus may pass through the egg. The disease apparently is more likely to occur in some areas of sericulture than in others. Thus, in certain areas of high elevation in France, notably in Ardèche, frequent outbreaks occur.

Control of the disease depends largely upon the maintenance of sanitary conditions in the silkworm-rearing rooms. Repeated and general disinfection during an extended suspension of all rearing operations is frequently required to bring about a complete eradication of the disease.

As we have already explained, gattine appears to be caused by a submicroscopic virus to which the bacterium *Streptococcus bombycis* Zopf (= *Micrococcus bombycis* Cohn) is a secondary invader. This combined etiology was first clearly elucidated by Paillot (1930*a*,*b*), who, because of the fact that gattine may occur in silkworms having very few bacteria of any kind in their alimentary tracts, suspected that perhaps something in addition to *Streptococcus bombycis* itself may be involved in bringing about the disease.

This suspicion was supported by the fact that the diarrheic intestinal contents of the infected insects cleared of bacteria by centrifugation were still capable of initiating the disease in healthy larvae. At first Paillot thought that perhaps he was dealing with some sort of cytotoxic product which elaborated in the intestinal tract and which "conditioned" the pathogenic action of the streptococcus. Further experiments, like the following example, however, convinced this French worker that he was actually concerned with an ultravirus which was the true primary cause of the infection and which, in addition, was characteristically accompanied by the action of *Streptococcus bombycis*.

Paillot observed that silkworms could be given gattine by inoculating them or feeding them with a small amount of the centrifuged intestinal contents from diseased larvae preserved for a year's time in sealed tubes. Similar results were obtained using intestinal contents held for considerable periods of time in a dried state. Such materials contained no living streptococci. He also noticed that, at the beginning of the disease, when nuclear lesions are first discernible in the midgut epithelium, the intestinal contents of the insect are practically devoid of microorganisms. Soon after this, of course, the streptococci appear and multiply rapidly, bringing about the characteristic symptoms of the malady.

The Virus of Gattine. The facts just related indicate that the virus of gattine apparently is fairly resistant to the effects of drying or long preservation. The fact that it can retain its potency in the dust of the rearing cages for long periods of time makes this source of infection an important one from the standpoint of the epizootiology of the disease. Information on the other properties of the virus is meager.

The fact that the intestinal contents of the infected silkworms lose their virulence when filtered through a Chamberland porcelain filter (L3–17) indicates that the virus is not readily filterable.

Dark-field examinations of centrifuged virulent intestinal contents reveals the presence of granules suspended in the liquid. According to Paillot, these granules (which he named *Borrelina flacheriae*) are similar to those seen in the blood of silkworms infected with the virus of jaundice and of those seen in *Pieris brassicae* (Linn.) infected with *Paillotella pieris* (Paillot). Such granules are not seen in the centrifuged intestinal contents of healthy silkworms or in the avirulent filtrates from diseased larvae. It is these granules which Paillot considers to be the parasitic elements of gattine. They apparently multiply in the epithelial cells of the alimentary tract of the silkworm.

Paillot discounts the possibility that the virus represents an invisible form of *Streptococcus bombycis,* since the latter does not always accompany the virus and, as will be explained later, at least one other bacterium may assume the secondary role of the streptococcus. He does, however, in some respects liken the relationship to that between the virus of hog cholera and the bacterium *Salmonella choleraesuis* (Smith).

Certain of the histopathological lesions produced in the intestinal epithelium of silkworms with gattine are produced by the virus alone. These may be demonstrated in larvae that have not as yet been invaded by the streptococcus. The streptococcic infection is nearly always manifested after the appearance of the characteristic nuclear lesions in the midgut epithelium.

The virus infection alone does not significantly alter the pH of the

intestinal contents of the diseased silkworm from that of the normal insects. Upon the invasion by the streptococci, however, the intestinal contents characteristically become more alkaline.

The Histopathology of Gattine. The histopathology of gattine has been studied by Paillot (1930*b*), who found that one of the simplest yet most reliable methods of differentiating gattine-diseased tissue from other types is by the use of Giemsa-stained impression smears. A portion of the silkworm's midgut is removed and opened; its inner face is then pressed against a clean glass slide several times in a different spot each time. By this procedure the epithelial cells of the midgut adhere to the slide without being noticeably distorted. The slide is then dried, fixed with methyl alcohol, and stained with Giemsa's solution for ½ to 1 hour. Such a preparation of the midgut of a gattinous silkworm shows the nuclei to be greatly hypertrophied (Fig. 169), staining a rose color and containing irregular granules. Sometimes large cracks appear to be present in the nuclear material.

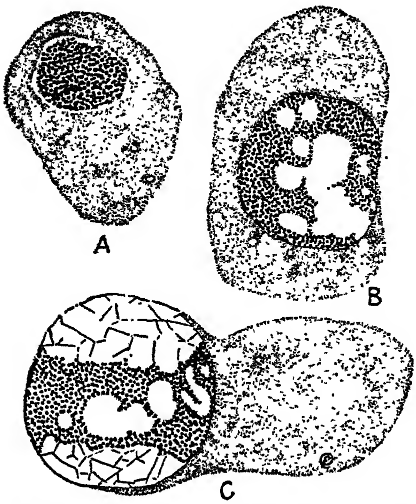


Fig. 169. Epithelial cells from the posterior third of the midgut of a silkworm suffering from gattine. Stained with Giemsa's solution. *A*. A normal cell. *B* and *C*. Pathological cells showing greatly hypertrophied nuclei. (*Redrawn from Paillot, 1930b.*)

The hypertrophied nuclei of the epithelium cells of the midgut of the diseased insects may also be seen in regular histological sections. In the posterior portion of the midgut, the enlarged nuclei are accompanied by curious alterations in the morphological structure of the chromatin and nucleoplasmic substance. The chromatin material may appear as a finely granular mass, or it may become gathered into irregular accumulations, or into rings. Eventually the contents of the nucleus are destroyed, and the nuclear area appears in sections as a lightly stained clear spot. The distal end of the epithelial cells becomes very vacuolated. The mitochondria, normally long and flexible and arranged in longitudinal rows in the basal part of the cell, break up into granules or into extremely short bodies of reduced diameter. The striated border is largely destroyed.

Histopathological changes may also be noted in the slender cylindrical cells of the anterior midgut. The nuclei move toward the distal part of

the cell, the mitochondria tend to become concentrated toward the outer border of the cells, and drops of secretion form abundantly. This last characteristic indicates the existence of a state of functional disturbances in the gut of the diseased insect which has been characterized by Paillot thus: hypersecretion in the anterior region of the midgut, accompanied by a cellular destruction of the wall and an accumulation, in the fore part

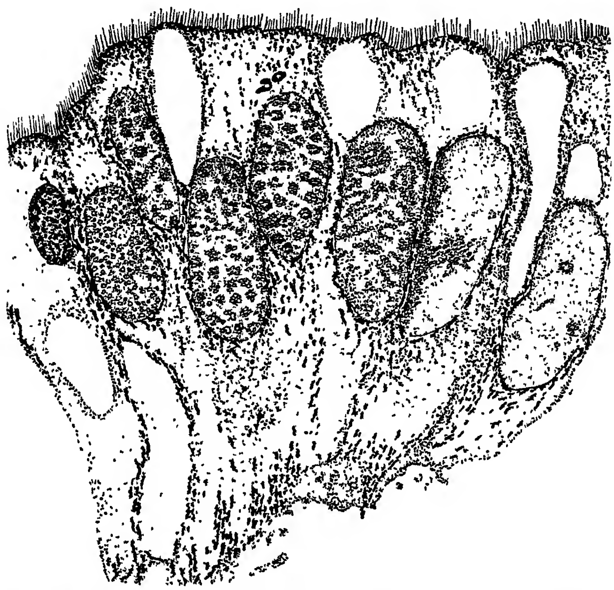


Fig. 170. Drawing of a longitudinal section through the posterior midgut of a silkworm suffering from gattine. To compare with normal epithelium, see Fig. 24*A*. Note alterations in the morphological structure of the chromatin and nucleoplasmic substance and in the appearance of the mitochondria. (*Modified and redrawn from Paillot, 1930b.*)

of the digestive tract, of the clear ropy secretion having a reaction slightly more alkaline than the normal secretion; want of an appetite; a more or less accentuated diarrhea.

The Streptococcus and Its Role in the Disease. It is generally assumed that the organism we now know as *Streptococcus bombycis* Zopf is the "ferment en chapelets de grains" seen by Pasteur in his investigations on the nature of flacherie in the silkworm. The bacterium has also been known by the name *Micrococcus bombycis* Cohn., but the fact that its element may occur in chains sanctions its placement in the genus *Streptococcus*.

Although *Streptococcus bombycis* apparently is but an accompanying or a secondary factor in gattine, its role is such that it merits our consideration of it as an important part of the etiology of the disease. It is a gram-

positive coccus of the enterococcus type, belonging to the streptococcus serological group D (Seelemann, 1942). The individual cocci are spherical or slightly oval in shape and measure approximately 0.9 micron in diameter. The chains, when they occur, are of variable lengths but usually measure from 5 to 12 microns. The organism grows well on most ordinary bacteriological media. Neither gelatin nor coagulated serum is liquefied, and certain carbohydrates are fermented to a slight degree while others are not attacked.

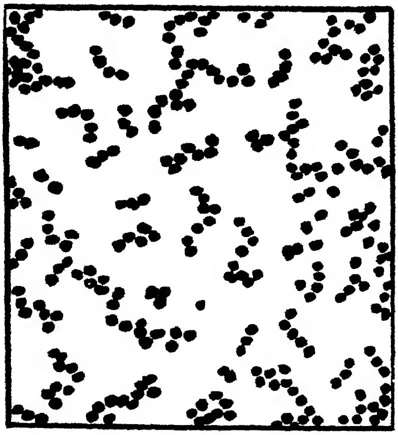


Fig. 171. Drawing of *Streptococcus bombycis* Zopf, the secondary invader in gattine, of which the primary cause is an ultramicroscopic virus.

Upon the inoculation of *Streptococcus bombycis* alone into the body cavity of a silkworm, a characteristic reaction and pathology result. According to Paillot, the streptococci are first actively phagocytosed by the leucocytes (micronucleocytes) until, 2 days after the inoculation, these blood cells contain large numbers of the bacteria. The lymphocytes also participate in this reaction to some extent. Three days after the inoculation fewer streptococci are seen in the phagocytes, although numerous granules may be present, indicating that the bacteria are in the process of being digested. The lymphocytes have a tendency to gather together to form veritable multinucleated plasmodia. About 2 days after inoculation, sections of the silkworm show the accumulation of large masses of streptococci in the vicinity of certain intra- and perivascular pericardial cells. These bacterial masses penetrate into the protoplasm of the cells but do not appear to cause much cellular damage. Analogous reactions occur in the area of the peritracheal cells. In no case are damaging effects observable, and the bacteria are apparently removed by digestion as in the case of the blood cells.

Some of the inoculated streptococci gather in masses along the sheaves of longitudinal muscle fibers of the external covering of the alimentary tract of the silkworm. The bacterial masses move on through the muscle cells, across the circular fibers of the middle layer, through the glandular epithelium, and finally pass through the gut epithelium into the intestinal lumen where they multiply rapidly in the gut contents. Peculiarly enough, apparently no serious damage is caused to most of the tissues through

which the streptococci passed on their journey. The infection does appear to cause some alteration of the epithelial cells of the midgut. Most of these changes concern the mitochondria and affect the secretory function of the cells, which are caused to secrete copious amounts of clear fluid. This slightly alkaline fluid rapidly fills and distends the anterior part of the midgut at the same time that the epithelial cells are undergoing a certain amount of destruction. It is this accumulation of fluid which gives the diseased insects the aspect of "clear heads," so frequently used in describing the appearance of gattinous silkworms.

Thus it is seen that *Streptococcus bombycis* plays an important part in the development of gattine, but it is not the principal cause. In the absence of the gattine virus the characteristic tissue lesions are not to be found, nor is the syndrome of gattine, as it is recognized by sericulturists, complete. These symptoms apparently are manifested only when the combination of streptococcus and virus occurs in the susceptible silkworm.

True Flacherie, or Flacherie of Pasteur

The historical aspects and terminology relating to true flacherie have already been discussed. We have also indicated that the same virus responsible for the initiation of gattine is the primary causative agent of flacherie. The etiology of flacherie differs from that of gattine in that, whereas the secondary invader in gattine is a streptococcus, that in true flacherie is a sporeforming bacillus, *Bacillus bombycis* auctt.

The general remarks we have made concerning the epizootiology of gattine in most instances apply to flacherie as well. The distribution of the two diseases is also approximately the same except that gattine occurs much more commonly than does true flacherie. Since the ultramicroscopic virus in both diseases appears to be the same, we shall not repeat here what has already been covered on this point in an earlier paragraph.

The symptoms of flacherie were well described by Pasteur, who conducted the first extensive scientific study of the disease. In a letter that he wrote to J. B. Dumas on June 3, 1867, Pasteur describes the symptoms of flacherie as follows: The bedding material is covered with silkworms, all having the full size for their age; but, strangely enough, these larvae are dead or dying, and they are so sluggish that their movements are scarcely noticeable, although their exterior appearance is so satisfying that it is necessary to touch and handle the dead ones to be certain that they are no longer living. If some have already crawled up into the bedding or heather, they stretch out on the stems and remain there without movement until death, or else they hang suspended downward, held up only by some of their prolegs. In these positions they become soft and

putrefy, assuming a blackish color in the space of 24 to 48 hours. Their bodies are then no more than blackish-brown sacs filled with bacteria that were first present in the contents of the insect's intestinal tract.

Pasteur, of course, had no reason to believe that the sporeforming bacillus he observed was not the cause of the disease. He suggested that the disease was brought on by either hereditary or accidental causes such as too great an accumulation of larvae of different instars, too high a temperature at the time of molting, poor ventilation, inclement weather, and improper food, factors that are still recognized as important contributing causes to outbreaks of bacterial dysenteries in insects.

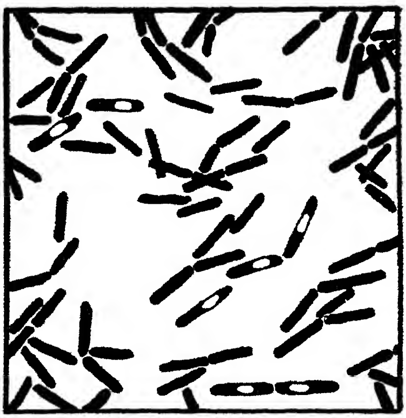


Fig. 172. Drawing of *Bacillus bombycis* auctt., the secondary invader in true flacherie, of which the primary cause is an ultramicroscopic virus.

The Role of *Bacillus bombycis* Auctt. in True Flacherie. The name *Bacillus bombycis* is surrounded by several nomenclatorial vagaries that confuse the picture considerably. Unfortunately Pasteur (1870), who discovered the organism, neither named nor precisely described it. He characterized it as being "des vibrions, souvent très-agiles, avec ou sans noyaux brilliants dans leur intérieur." In 1891 Macchiati gave the name *Bacillus bombycis* to a sporeforming organism he found in the larva, pupa, and adult of the silkworm. There are several reasons for believing that Macchiati's organism is not the same as Pasteur's "*vibrion à noyau,*" two of which have been pointed out by Paillot (1930*b*): (1) Pasteur's bacillus is not cultivable on ordinary bacteriological media, whereas Macchiati apparently had no difficulty in thus growing his organism; (2) Pasteur's bacillus is readily decolorized in using Gram's method of staining, while Macchiati's organism apparently is not. According to Paillot, these two criteria would also serve to differentiate Pasteur's organism from Ishiwata's *Bacillus sotto*, a bacterium found in association with flacherie in Japan and similar to another sporeformer found in diseased silkworms in France. Also thus eliminated would be the possibility suggested by Sawamura (1905) that the organism with which we are concerned is a strain of *Bacillus megatherium* De Bary.

According to accepted procedures of nomenclature, Macchiati's original use of the name *Bacillus bombycis* for the cultivable sporeformer he isolated would preclude its use for Pasteur's bacillus. In light of the

dissimilarity between Macchiati's and Pasteur's organisms, however, Paillot (1930*b*) has seen fit to retain the name *Bacillus bombycis* for Pasteur's bacillus and to suggest that another name be given to the species isolated by the Italian author. Throughout most of the recent literature on flacherie the name *Bacillus bombycis* has been used to designate the sporeformer observed by Pasteur and subsequent workers. Macchiati's bacillus has had no additional work done on it, and a guess might be that it was one of the common more or less saprophytic sporeformers known today. Paillot's procedure in using the name *Bacillus bombycis* for Pasteur's organism makes a homonym of the name and is contrary to the accepted rules of nomenclature. It therefore becomes a question of accepting it on the basis of common usage or of proposing a new name for it. The latter procedure would no doubt be the more correct one, but whether a new name would now be accepted by workers in this field is another matter. Since nearly all users of the name *Bacillus bombycis* have used it in referring to Pasteur's organism (and Macchiati apparently thought he was dealing with Pasteur's organism), its acceptance in its present form would probably be favored by most authorities. It may therefore be properly referred to as *Bacillus bombycis* auctt.

The name *Bacillus bombycis* has also been used by Chatton (1913) for a small nonsporeforming gram-negative rod. Paillot (1933) used the name *Bacillus bombycis nonliquefaciens* for a nonsporulating bacterium. In both these cases it is clear that the names are not valid, since both are antedated by Macchiati's use of the name for the sporeformer. Whether any of the bacteria just mentioned is Joly's (1858) "*Vibrio Aglaiae*," apparently the first bacterium described from diseased silkworms, is uncertain.

According to Paillot (1930*b*), *Bacillus bombycis* is incapable of multiplying in the intestinal contents of normal healthy silkworms. On the other hand, if the digestive tract is in a state of abnormal function, such as is brought about by the presence of the virus, the bacillus rapidly multiplies and causes the lesions, more or less modifying those caused by the virus itself, characteristic of true flacherie. An acrid disagreeable odor, due to the volatile acids formed by the fermentation of the intestinal contents and given off by the ailing larvae, is characteristically present in nearly all outbreaks of the disease. Pasteur, in his writings on the disease, describes this odor and mentions that a brood of only 100 infected silkworms is able to give off, from a basket containing them, a pronounced odor. The same odor is not present in the case of gattine.

The histopathological lesions caused by the multiplication of *Bacillus bombycis* are not so distinct as are those provoked by the ultravirus alone. In general, the lesions caused by the virus are aggravated by the bacillus, especially those relating to the mitochondria and the epithelial cells of

the posterior region of the midintestine as well as those of the anterior and middle regions. The nuclear lesions caused by the ultravirus itself are no longer limited to the posterior region, as explained in our discussion of gattine, but extend into the middle region. After the bacillus gains the foothold made possible by the initial invasion by the virus, it appears to cause a modification of the chemistry of the epithelial cells of the middle region of the midgut, rendering them sensitive to infection by the ultravirus. Subsequent to his early observations on the histopathology of the disease, Paillot (1941*b*) noticed that in silkworms taken from one particular diseased brood, the nuclear lesions extend to all the cells of the midintestine, including those of the anterior midintestine. Since he had previously considered the cells of the anterior midintestine as relatively resistant to the virus, Paillot interprets this finding as evidence that one of the fundamental properties of the virus has been modified and that this constitutes a true natural mutation. That such a conclusion is warranted on the basis of the meager evidence presented is questionable. Before ascribing the modified histopathological changes entirely to the virus, further consideration should be given the various environmental and host factors possibly involved.

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CHAPTER 12

PROTOZOAN INFECTIONS

The preceding chapters have been concerned with what are generally considered plantlike organisms—bacteria, yeasts, and fungi—and viruses. We come now to those one-celled (or, as some prefer to say, noncelled) animals known as "protozoa," and the infections in one group of animals (insects) as caused by another group of animals (protozoa) will be considered.

Although structurally comparatively simple, a protozoan nevertheless is, in a sense, functionally as complete an organism as is a metazoan, performing all the essential life processes of an animal. An amoeba is generally considered to typify one of the simplest of these forms of life and is frequently used as a representative example of a protozoan. In comparison with the amoeba, some kinds of protozoa are extremely complex in their manner of life and reproduction; basically, however, all protozoa are similar in these respects.

The amoeba, like nearly all protozoa, is of microscopic size, and its dimensions are usually measured in microns. Structurally there is an outer layer of protoplasm, called the "ectoplasm," which usually contains no formed bodies and is somewhat glassy in appearance, and an inner portion, granular and containing inclusions or organelles, called the "endoplasm." This contains a nucleus similar in structure and function to that of higher organisms. Frequently, within the endoplasm, there is also a kind of liquid-filled "bubble," known as a "contractile vacuole," the function of which is to preserve the water balance in the cell. It slowly enlarges, moves to the surface of the cell, bursts, and discharges its contents.

Some protozoa feed on other microorganisms, such as bacteria and algae, which, enclosed in a food vacuole, are taken into the interior of the cell where they are dissolved, digested, and absorbed into the protoplasm. This type of nutrition is called "holozoic nutrition" and is typical of all animals in that the ingested organic matter is produced or synthesized by other organisms and then taken into the body of the animal, digested, and assimilated. Saprozoic nutrition, the absorption of dissolved nutrient materials by diffusion through the body surface, is characteristic of the most important groups of entomogenous protozoa. A few examples of

holophytic nutrition, in which food is synthesized in the body, also exist among protozoa.

Reproduction may be quite complex when considered in all its aspects and life-cycle relationships, but basically it may be rather simple. Thus in an amoeba the nucleus divides into two parts in what is known as "mitotic division"; *i.e.*, the chromatin in the nucleus first becomes arranged in chromosomes that divide and separate, after which the whole cell divides into two parts, each with its own nucleus. Thus binary fission of the protozoan is accomplished. Other processes may intervene; resting forms known as cysts (or, in the case of some protozoa, spores) may develop. These forms are surrounded by thick walls, and within them the nucleus may divide a number of times. When brought into favorable conditions, the cyst or spore germinates and new cells appear.

Classes of Protozoa. The amoeba to which we referred is only one kind of organism to which the name "protozoa" is applied. Some authorities consider the amoeboid-flagellate group of organisms as comprising a more primitive group, phylogenetically distinct from the other forms, thus dispensing with the idea of the single large phylum Protozoa. For the present, however, probably the safest procedure is to conform to the orthodox concept by which the phylum Protozoa is considered as a separate subdivision of the animal kingdom. This phylum is generally presented as consisting of five classes. The names of the five classes, which are broadly separated on the basis of the structures they may possess for locomotion, are Mastigophora or Flagellata (which move by flagella), Sarcodina (which move by pseudopodia), Sporozoa (which have no special means for locomotion), Ciliata (which move by cilia), and Suctoria (which first have cilia and later tentacles).

For the sake of convenience and orderly discussion we shall, in this chapter, consider the protozoan infections of insects according to the classes to which the causative protozoa belong.

As a group, most protozoa are free-living, *i.e.*, they live freely in nature and are not dependent upon living in or on another organism or host. A large number, however, do live in association with higher organisms and may be parasitic on them.

Parasitic Protozoa. Although protozoa may parasitize animals of almost any phylum, we are here concerned principally with those which parasitize and cause diseases in insects (Arthropoda).

The intimate association of a protozoan with an insect may indicate any of a wide range of biological relationships. A protozoan may be definitely beneficial as in the case of those protozoa which live in the gut of the termite and without which the insect cannot continue to live. A protozoan may establish a commensal relationship with the insect and

be neither helpful nor detrimental. These types of relationship and their variations have been considered in an early chapter. The association that here interests us most is that in which the protozoan is parasitic (pathogenic) or otherwise definitely detrimental to the host insect. It is to this relationship that we may properly apply the term "protozoan infection" or "protozoan disease," as the case may be.

A protozoan infection may be of epizootic proportions, or it may represent a purely localized disturbance caused by the invasion of a protozoan into some cell or tissue of the insect's body. In nature, even the protozoan epizootics are usually fairly well limited to small areas or to small percentages of the host population.

Unlike most of the diseases caused by bacteria and viruses, those incited by protozoa, with certain exceptions, are slow in developing. When a virulent bacterium invades the body cavity of an insect, the infection usually develops rapidly, with the death of the host an expected consequence. In the case of a protozoan infection, however, the diseased condition may become chronic in nature. To be sure, in many instances the infection is highly fatal, such as is the case with the microsporidian disease pebrine in the silkworm. In other cases, however, a protozoan-infected larva may pupate and complete its development as an adult without succumbing to the effects of the disease. Such a situation occurs with some of the coccidian infections, for example.

The epizootiology of a protozoan disease may be entirely different from that of one caused by a bacterium, fungus, or virus. Methods of transmission, for example, may be limited to that of ingestion of ripe cysts or spores, or to transovarial passage of the infectious agent through the egg to the next generation, or to transmission by the ovipositor of a parasitic insect. Many pathogenic or parasitic protozoa form resistant spores or cysts that enable them to withstand unfavorable environmental conditions while awaiting entrance into a host. These spores may not be so durable as the spores of certain bacteria, but they do enable the organism to wait out periods between hosts better than if no such mechanism of resistance were provided. This characteristic should also be valuable in making it easier to distribute protozoa artificially in attempting to use these organisms for purposes of biological control.

Most protozoa, once they enter the digestive tract of a susceptible host, are fairly certain of being able to continue their invasion of the host's tissues. This is not always the case with bacteria, which may enter the gut of an insect in considerable abundance, but because of their low initial invasive power an infection does not result. That is to say, pathogenic protozoa, generally, have a much greater stability as concerns their invasive powers than do bacteria that frequently require primary causes

to act before they are capable of bringing about a frank infection. To be sure, predisposing factors are also important in certain protozoan diseases. Numerous destructive factors act on protozoa in the intestines of insects, and some species (certain microsporidia) are entirely unable to invade the body cavity of a susceptible host through the wall of the alimentary tract. In the use of protozoa as a means of insect control, however, there appear to be means of getting around these difficulties more easily than seems to be the case with certain bacteria.

Protozoa in Biological Control. The artificial distribution of protozoa for the purpose of controlling insects biologically has, for the most part, been tried in only a halfhearted spirit. The reasons for this are understandable when it is realized that like viruses, but unlike bacteria and fungi, most parasitic protozoa are not readily cultivable on artificial media. Accordingly, the mass production of protozoa for field distribution is attended by problems essentially similar to those associated with viruses. With most of the protozoa associated with insects, insectary methods are probably the most practical for producing large numbers of these microorganisms; *i.e.*, an appropriate host is reared in large numbers, these host insects are then mass-infected, and the resulting diseased insects are prepared for field distribution. Means of distributing the protozoa in the field so as to obtain effective results in destroying agriculture pests for the most part still remain to be worked out.

That protozoa are effective in the natural control of certain pests in certain localized areas there is no doubt. It is questionable, however, whether Paillot's (1928) statement that protozoa are more important in this regard than are bacteria can be substantiated by facts. Actually, sufficient data have not been gathered to determine by any means the true role of protozoa in the natural destruction of insects. It should be pointed out, however, that organisms such as protozoa may be an important factor in the destruction of insect pests without necessarily being able to bring that pest under actual control.

Examples and the possibilities of the biological control of insects by means of protozoa will be cited throughout the present chapter and in Chap. 14.

MASTIGOPHORA
(Flagellata)

In Chap. 4 the flagellates that are associated with healthy insects were mentioned briefly. It was also explained that extremely few flagellates cause actual infection, disease, or pathological changes in insects. The student should not be confused by the fact that the word "infection" is applied loosely throughout most of the literature to indicate the mere

presence and development of the flagellate within the alimentary tract of an insect. This usage of the term probably should be discouraged and the word "infection" reserved to indicate the invasion of the tissues of the insect's body by living pathogenic microorganisms in such a way that their growth and toxin production injure the tissues or cells involved. It seems likely, however, that the word will continue to be used loosely in much of the literature.

FLAGELLATE INFECTIONS

When a flagellate leaves the intestinal canal of an insect and penetrates into the body cavity, we have reason to suspect that such an invasion will probably bring at least some harm to the host. Such detrimental results, however, are not always apparent. Several species of flagellates have been found in the body cavity as well as in the salivary glands and other tissues of insects without their effect on the host being determined. In other cases, there is a slight indication that the parasite has at least some pathogenicity for certain hosts. For example, *Leptomonas pyrrhocoris* L. & D. has been observed in the gut, body cavity, and salivary glands of the plant bug *Pyrrhocoris apterus* (Linn.). Experimentally, larvae of certain flies, beetles, moths, and bugs are susceptible to this flagellate, which develops well in their body cavities but usually does not kill them. Other examples could be cited. Whether such situations constitute true infections is difficult to decide. It is possible that a flagellate introduced into the hemolymph of an insect could live and multiply without greatly disturbing its host, but this would seem unlikely in those cases in which the protozoan developed to great abundance.

The flagellate just mentioned, *Leptomonas pyrrhocoris* L. & D., when inoculated into the body cavity of larvae of the wax moth, *Galleria mellonella* (Linn.), usually produces a fatal infection. Zotta (1921) observed that the larvae often succeed in forming pupae that remain infected but that these pupae rarely became adults. Of particular interest is the fact that the flagellates inoculated into the body cavity of this insect make their way through the musculature and epithelium of the digestive tract into the lumen of the gut, more or less reversing the process as it occurs in the flagellate's original host, *Pyrrhocoris apterus* (Linn.). On the other hand, the mealworm, *Tenebrio molitor* Linn., retains the infection throughout all stages from larva to adult. In the phasmid *Carausius morosus* Br., the flagellates disintegrate or become degenerate, indicating a strong humoral immunity reaction on the part of the insect. In most of the susceptible insects, phagocytosis, or cellular immunity, is fairly strong; the leucocytes tend to form accumulations known as "giant cells."

Some flagellates have a habit of attaching themselves to the intestinal

lining of their host with the result that the border of the gut wall is abnormal in appearance. An example of this is the infection of the corn borer, *Pyrausta nubilalis* (Hbn.), by a flagellate discovered in this insect in France by Paillot (1928), and named *Leptomonas pyraustae* Paillot. The protozoan occurs in the Malpighian tubes of the corn borer as well as in the alimentary tract.

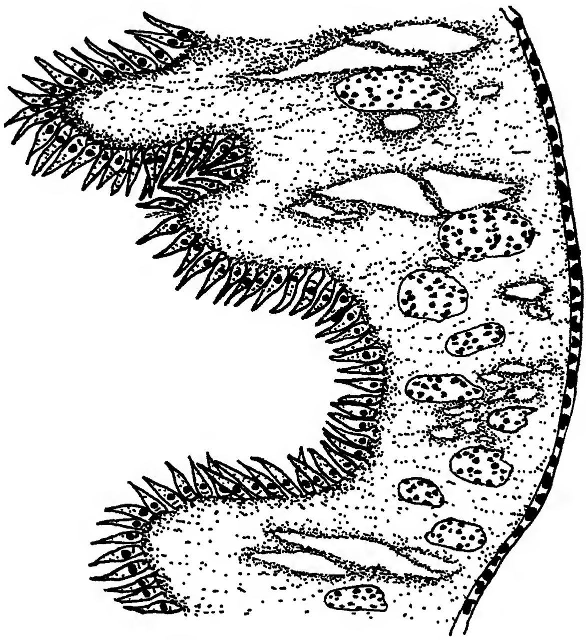


Fig. 173. Section through the intestine of the larva of the European corn borer, showing presence of *Leptomonas pyraustae* Paillot along edge of epithelium. (*Adapted from Paillot, 1928.*)

The infected corn borers show no outward symptoms, and they are indistinguishable from uninfected individuals. Internally, the Malpighian tubes are grayish in color and slightly hypertrophied. In the midgut the flagellates attach themselves by their anterior ends to the epithelium, and no deeper injury to the host cells can be seen. Occasionally a specimen is found in which the flagellate occurs in the hemolymph as well. Paillot found only a small percentage (4 out of 620) of corn-borer larvae harboring the protozoan.

A similar association is known to occur in the dog flea, *Ctenocephalides canis* (Curtis), which harbors *Leptomonas ctenocephali* Fant. Several species of flagellates attach themselves to the walls of the intestinal tract and Malpighian tubes of drosophila flies. Depending upon various

circumstances, the flagellates may occur within the peritrophic membrane of the gut or outside this membrane but attached to the cells of the gut wall. A considerable number of other examples exist in which the flagellates are attached to the walls of the gut or Malpighian tubes of an insect.

Some species of flagellates that cause infection in vertebrates also invade the cells of their insect vector during their development in the invertebrate. Thus *Trypanosoma lewisi* (Kent), which causes a mild type of infection in the rat, invades the cells of the stomach wall of the flea, *Nosopsyllus fasciatus* (Bosc d'Antio), which transmits it. As the flagellate penetrates the cell, a vacuole is formed about the trypanosome, which then undergoes further development and multiplication. The invaded cell frequently is reduced to a mere membrane enclosing actively moving organisms. Eventually, the cell ruptures; the trypanosomes escape into the stomach of the flea, and from there they may invade other epithelial cells and repeat the process. This example simply illustrates a type of cellular pathology that one may find in insects that are vectors of flagellates that cause infections in other animals.

SARCODINA

From the standpoint of their pathogenicity for insects, the most important protozoa in the class Sarcodina are those of the order Amoebida (commonly referred to as "amoebae" or "amebas"). The Amoebida may, in brief, be thought of as a group of protozoa which have bodies without cuticles, although at times they may be enclosed within a cyst wall, and which have a peculiar method of locomotion by means of pseudopodia. The cytoplasm is differentiated into fairly distinct zones of ectoplasm and endoplasm. The parasitic amoebae usually have no contractile vacuole. The various genera and species of amoebae are generally distinguished from each other by the structure of the nucleus and the nature of the cysts. Asexual reproduction is generally by binary fission. Encystment is common.

AMOEBIC INFECTIONS

Only a few species of amoebae pathogenic for insects have been reported. As with the flagellates, most of the amoebae associated with insects do not cause much harm to the tissues of their hosts, and some of them are true commensals. Species representing the latter relationship have been dealt with in Chap. 4.

Amoebic Disease of the Honeybee. One of the most important diseases of insects known to be caused by an amoeba is that of amoebic disease (sometimes written "amoeba-disease") of the adult honeybee, *Apis mellifera* Linn. The disease occurs in Europe, particularly in Germany,

Switzerland, and Great Britain, and has also been reported in the United States (Bulger, 1928).

Amoebic disease of the honeybee, probably first seen in Europe in 1916 by Maassen, is caused by an amoeba which Prell (1926) described and named *Malpighamoeba mellificae*. This organism was subsequently placed in the genus *Valkampfia*. It causes a heavy parasitization of the insect's Malpighian tubes which, in extreme cases, are somewhat distended and have their function completely disrupted. The epithelial cells of these structures may be injured. In general appearance the tubes have a somewhat glassy aspect. The tubes become filled with hundreds of encysted amoebae which are more or less spherical in shape and from 5 to 8 microns in diameter. As pointed out by Morison (1931), when infected tubes are examined in water under a microscope, the cysts look like pearls inside the tubes or scattered about the preparation if the tubes are broken. They are usually distinguishable from cells of yeasts and fungi by their thicker walls and by their size and shape. Amoebic disease frequently occurs concurrently with nosema disease, but the amoebic cysts may be distinguished from the microsporidian spores by the smaller size and elliptical shape of the latter. The large intestine of the bee may also contain large numbers of cysts, which eventually are discharged from the insect with the dejecta.

Transmission of the parasite takes place when the cysts are ingested by susceptible bees.

The disease is most severe in the spring of the year. If it occurs coincident with nosema disease the combination may easily exterminate the colony. If only the amoebic disease is present, the colony may survive the spring and yield a profitable amount of honey, but the disease is likely to reappear the following spring in a more severe degree.

The principal symptom of a diseased colony is the gradual decrease of the number of bees. Unlike the situation in certain other infections, bees afflicted with amoebic disease usually do not die in close proximity to the hive. Death of an infected bee may result from the loss of function of the Malpighian tubes or from cold temperatures outside the hive which are ordinarily resisted by healthy bees.

Since very little is known about the details of the disease, not much in the way of specific treatment has been forthcoming. Morison (1931) has suggested the following general measures: practice good beekeeping by wintering strong colonies with ample well-ripened stores in weatherproof hives; encourage breeding as much as possible in the spring; see that the queen is prolific; keep the apiary as clean and dry as possible; keep the water supply as clean as possible; gather and burn dead bees; scrape the frames and the interior of the hive and burn the scrapings; sterilize

the woodwork of the hive and broodbox with the flame of a blowtorch, or use a 1 or 2 per cent solution of carbolic acid or other strong disinfectant.

Amoebic Disease of Grasshoppers. In 1936 King and Taylor reported the presence of a parasitic amoeba, which they named *Malpighamoeba locustae*, in the Malpighian tubes of grasshoppers of the genus *Melanoplus*

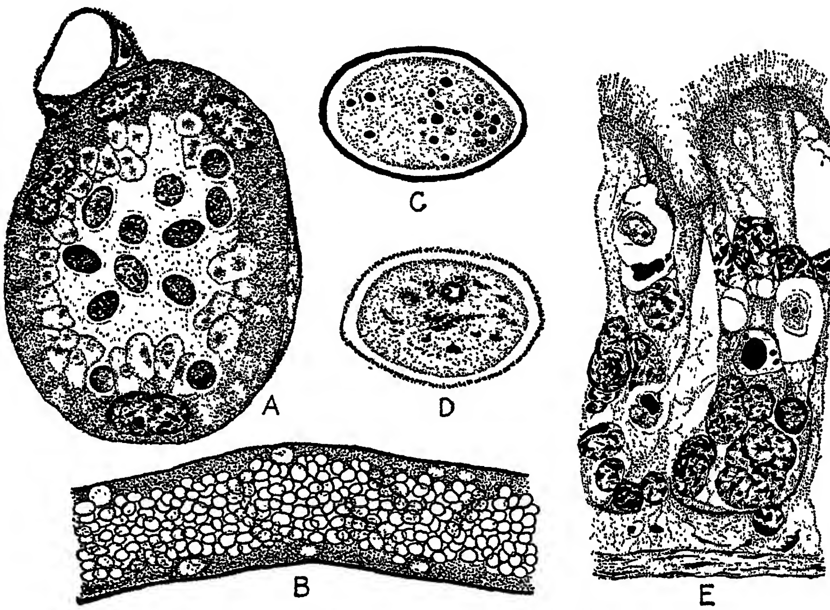


Fig. 174. A parasitic amoeba, *Malameba locustae* (King & Taylor), in grasshoppers (*Melanoplus*). *A.* Cross section of a Malpighian tube of grasshopper infected with the amoeba, showing cysts and trophozoites. The brush border of the tube has been destroyed. *B.* A short section of a Malpighian tube the lumen of which is packed with cysts. *C.* Drawing of a living cyst. *D.* Drawing of a cyst from fixed material. *E.* Trophozoites in the epithelial cells lining the midgut of *Melanoplus differentialis* (Thos.). Stained with iron hemotoxylin. (*Redrawn from King and Taylor, 1936, and Taylor and King, 1937.*)

(*M. differentialis* (Thos.), *M. mexicanus mexicanus* (Sauss.), and *M. femur-rubrum* (DeG.)). Later Taylor and King (1937) reconsidered their taxonomic allocation of the organism and proposed a new genus, *Malameba*, for it. They found further that the parasites occur not only in the lumen of the Malpighian tubes, as they had previously reported, but that trophozoites may be observed also within the epithelial cells of the midgut and in those lining the gastric caeca. Within these cells the amoebae are not restricted to any particular region, and they are surrounded by a clear vacuole.

The parasitized Malpighian tubes are usually found to be swollen,

more or less glassy in appearance, and packed with cysts. As the tubes increase in diameter, their epithelial cells become thinner and finally disappear altogether. Eventually, the swollen tubes, which are under great pressure, may burst and liberate the cysts into the hemocoele, where they are surrounded by hemocytes. Some of the cysts are carried to various parts of the body and may be seen embedded in dark masses in the fat body and in the muscles of the head and thorax. Upon the bursting of two or more adjacent Malpighian tubes there are frequently formed globular, tumorlike swellings that may reach a size of over 1 millimeter in diameter. These formations may result from the hypertrophy of a single tube, but usually it is from the consolidation of several tubules into a mass of tissue, surrounded by muscle and other cells. The interiors of these structures finally disintegrate, leaving the cysts embedded in a dark-brown matrix.

The trophozoite, or vegetative, stage of *Malameba locustae* (King & Taylor) has one nucleus and averages from 5 to 10 microns in diameter. The protoplasm is hyaline and contains from 8 to 30 highly refractile globules. Locomotion is by wavelike hemispherical pseudopodia and occasionally by filose pseudopodia. No contractile vacuole is present. In division stages the nuclear membrane disappears, the karyosome breaks down, and a spindle is formed with chromosomes on an equatorial plate. King and Taylor did not observe any plasmodialike multinuclear forms. The cysts are uninucleate, oval, circular in cross section, and from 8.5 to 10.0 microns long by 4.6 to 6.2 microns wide. The cyst wall is thick and hyaline. There are often a prominent vacuole and one or two rodlike inclusions in the cytosome.

The symptomatology of the disease varies with the degree or severity of infection. A light parasitization may yield no visible symptoms whatever. As the parasites become more numerous, the insect becomes increasingly sluggish and loses its appetite. The loss of the Malpighian-tube function probably prevents the proper excretion of toxic substances, which causes a disruption of the insect's normal metabolism. In advanced stages of the infection, the grasshopper enters a comatose condition in which it exhibits a marked inability to remain in an upright position. Just before the insect dies, the muscles of the jumping legs undergo tetanic twitches. The last noticeable movements appear to be those of the mouthparts. Infected nymphs show symptoms similar to those seen in adults. If the parasitization is heavy, the nymph may die before the adult stage is reached; this usually does not take place, however, until the fifth instar. If the insect is able to survive until the last molt, this period is completed with great difficulty, if at all, and in a considerable number of cases the insect comes through the molting process in a crippled condition.

Transmission of *Malameba locustae* takes place through the ingestion of food contaminated with cysts previously discharged from an infected grasshopper along with its feces. Between 2 to 4 million cysts are egested per day by infected male and female grasshoppers. The interval required for the development of cysts in the Malpighian tubes from the time the insect is exposed to an infective feeding is usually from 14 to 18 days. At least 37 species of grasshoppers (Acridinae, 5 species; Oedipodinae, 14 species; Cyrtacanthracrinae, 18 species) have been found to be experimentally susceptible to the parasite; only a few species appear to be insusceptible.

Malameba locustae does not appear to be common in grasshoppers collected in nature; at least Taylor and King (1937) did not find it so. In examining 633 specimens collected in Iowa, these workers found only two individuals infected with the parasite. An attempt was made to increase the intensity of the infection in the field. To do this, Taylor and King collected feces from contaminated cages and thoroughly mixed them with bran and a small amount of molasses. The mixture was scattered along roads and fences over an area not exceeding 100 square feet at three different places not far from Iowa City. Eight weeks later grasshoppers were collected within a radius of 20 or 30 feet of the area where the cysts had been spread. Upon examining the Malpighian tubes of these insects, it was found that out of a total of 422 individuals, 20 (4.74 per cent) were infected. Thus it would appear that the intensity of infection in nature can be increased. The figures cited are only relative, however, since there was no way of knowing how many infected grasshoppers had left and how many of those collected had just entered the area. A year later no infected individuals were found in the seeded area.

Other Amoebic Infections. Among most other amoebae parasitic in insects, very little is known of their actual pathogenicity or of the pathological changes they bring about in the tissues of their hosts. Accordingly, we are able to give here only brief mention of an example or two of these protozoa.

In 1917 Keilin described an amoeba that he found parasitizing larvae of the winter gnat, *Trichocera hiemalis* Meig., collected in Paris. Later he observed the parasite in the same insect and in *Trichocera annulata* Meig., collected in England. He gave the protozoan the name *Entamoeba mesnili*; this was later changed to *Dobellina mesnili* (Keilin) by Bishop and Tate (1939). Whether the parasite is ever capable of actually penetrating and destroying the tissues of the insect has not been determined with certainty. It is known to live in the lumen of the larval gut, mostly in the annular space between the peritrophic membrane and the gut epithelium. Some amoebae may occur within the peritrophic membrane along with the

food of the larva. Sometimes the amoebae are present in such large numbers that they form dense masses that completely fill the annular space.

SPOROZOA

None of the other classes of protozoa includes as many forms pathogenic for insects as does the class Sporozoa. For this reason, and because the spore stage of these organisms is rather resistant to adverse environmental conditions, certain of the Sporozoa perhaps offer a greater promise for use in methods of biologically controlling insects than do all other groups of protozoa combined. From the standpoint of insect pathology, therefore, it is necessary that we give particular attention to this group and treat it in considerable detail.

All members of the class Sporozoa are parasitic in habit and form spores in some stage of their development. These spores are small resistant bodies, each having a firm envelope or capsule within which are protectively enclosed one or more parasitic microorganisms awaiting the germination of the spore before continuing their development in the body of a suitable host. Most Sporozoa have two phases in their life cycles, one asexual (schizogony) and one sexual (sporogony). Except as gametes, they possess neither cilia nor flagella, and for the most part, except in certain stages, they are not actively motile. Their hosts are in every animal phylum.

Although some authorities divide the Sporozoa simply into two subclasses, Telosporidia and Neosporidia, most modern authors separate them into three such groups: Telosporidia, Acnidosporidia, and Cnidosporidia. Although the largest number of entomophilic species are in the Telosporidia, the most important from the standpoint of actual virulence for insects are included in the subclass Cnidosporidia. The latter are separated from those in the other two groups by the fact that the spore possesses a polar filament; the spore of those in the other two groups is simpler in structure and is without a polar filament. Only the entomogenous members of these groups will be considered here; those of the subclass Telosporidia will be treated first. These include such forms as the gregarines and the coccidia.

GREGARINE INFECTIONS IN INSECTS

Perhaps because of their large and conspicuous size the gregarines were the earliest Sporozoa to be observed and studied. Some historians state that Redi may have seen a gregarine in 1708, but apparently there is no doubt that Cavolini, in 1787, described and figured a gregarine from the glandular appendages of the stomach of the crustacean *Pachygraspus marmoratus* Stim. It remained, however, for Dufour (1828) to recognize the group as such. He established the generic name *Gregarina* and con-

sidered the organisms to be a peculiar group of worms related to the trematodes. It is of interest to note that Dufour made his observations on gregarines associated with insects and that they came about as a result of his study of insect anatomy. A few years after Dufour's report appeared, von Siebold, in 1839, contributed significant information on the group, and during the years around 1900, Léger, Schneider, and others made important observations that finally brought the group into its own as a distinct category of protozoa.

Most of the literature on gregarines is systematic in character. Unfortunately comparatively little is known concerning the physiology of these protozoa; still less is known about the biological relationships between them and their hosts. Most authorities consider them to be parasites that are relatively well tolerated by their hosts. Some workers have considered them to be distinctly beneficial. That they may at times be more harmful than beneficial, however, is indicated by the fact that they do destroy the epithelial cells to which they are attached in the gut of the host. It is also possible that they may bring about a general debilitating effect on the host, perhaps decreasing its general activity or its reproductive powers. There is little question, however, that they are properly included in a book on insect pathology.

Systematic and Morphological Considerations

The order Gregarinida is generally separated into two suborders: Eugregarinina and Schizogregarinina. The first of these is the larger group, and its members do not undergo asexual reproduction, or schizogony, but multiply sexually by sporogony. Schizogregarinina, on the other hand, undergo both sporogony and schizogony.

Eugregarinina

Life History. Soon after an appropriate host ingests an eugregarine spore the action of the host's digestive juices causes the spore to liberate the very small falciform sporozoites. Each sporozoite enters an epithelial cell of the intestinal wall where it grows at the expense of the host cell. It has been postulated, but not proved, that penetration by the sporozoite is effected by the parasite's secretion of a toxin that lowers the resistance of the cell wall. As soon as the sporozoite, within the host cell, begins to absorb nourishment and grow, it becomes a trophozoite. The developing trophozoite soon leaves the host cell to which it frequently remains attached for a time by a special organelle of attachment (the epimerite). Later, after the epithelial cell is destroyed or when it no longer furnishes sufficient nourishment or when its activity causes it to release its hold, the trophozoite becomes detached from the host cell and moves about in the

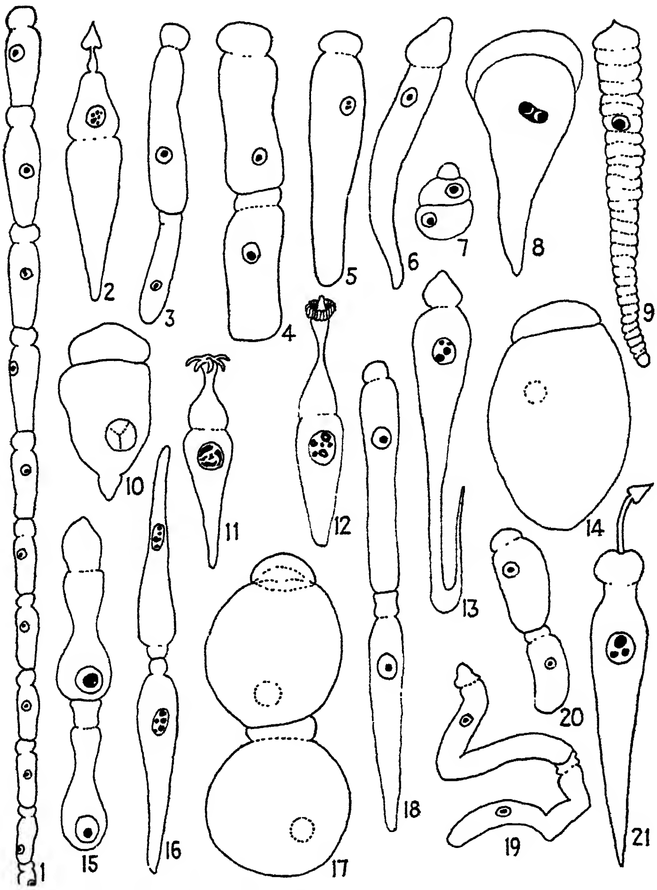


Fig. 175. Various types of gregarines (sporadin or sporont stage) from insects, mostly from the intestinal tracts of these animals. Not necessarily according to scale. 1. *Hirmocystis polymorpha* Léger from *Limnobia* larva, associated in linear fashion. 2. *Pileocephalus heeri* Köll. from *Phryganea* larva. 3. *Gregarina cetoniae* Foerster from *Cetonia* larva, in syzygy. 4. *Gregarina coelomica* Foerster from the body cavity of *Pyrochroa* adult, in syzygy. 5. *Lophocephalus insignis* (Schneider) from *Helops*. 6. *Actinocephalus notiophili* Foerster from *Notiophilus*. 7. *Didymophyes rotunda* Foerster from *Onthophagus*. 8. *Actinocephalus dytiscorum* (Frant.) from *Dytiscus*. 9. *Taeniocystis mira* Léger from *Ceratopogon* larva. 10. *Actinocephalus digitatus* Schneider from *Chlaenius*. 11. *Ancyrophora uncinata* Léger from *Dytiscus* and other insects. 12. *Asterophora elegans* Léger from *Phryganea* and *Sericostoma* larvae. 13. *Cometoides capitatus* (Léger) from *Hydrous* larva. 14. *Gregarina blattarum* von Siebold from *Blatta*. 15. *Gregarina lagenoides* (Léger) from *Lepisma*, in syzygy. 16. *Stylocephalus bahli* Misra from *Gonocephalum* adult, in syzygy. 17. *Gregarina statirde* Fern. from *Statira*. 18. *Gregarina longa* (Léger) from *Tipula* larva, in syzygy. 19. *Gregarina marteli* Léger from *Embia* larva. 20. *Gregarina katherina* Watson from *Coccinella*. 21. *Stylocephalus indicus* Misra from *Opatroides*. (*After figures by Léger, 1892; Kamm, 1922; Foerster, 1938; and Misra, 1941.*)

lumen of the gut. The epimerite, being no longer useful, drops off, and the parasite is now called a "sporadin" (sporont). In this easily recognizable stage the gregarine is usually large and vermiform. It is this form which is frequently seen attached to one end of the body of another

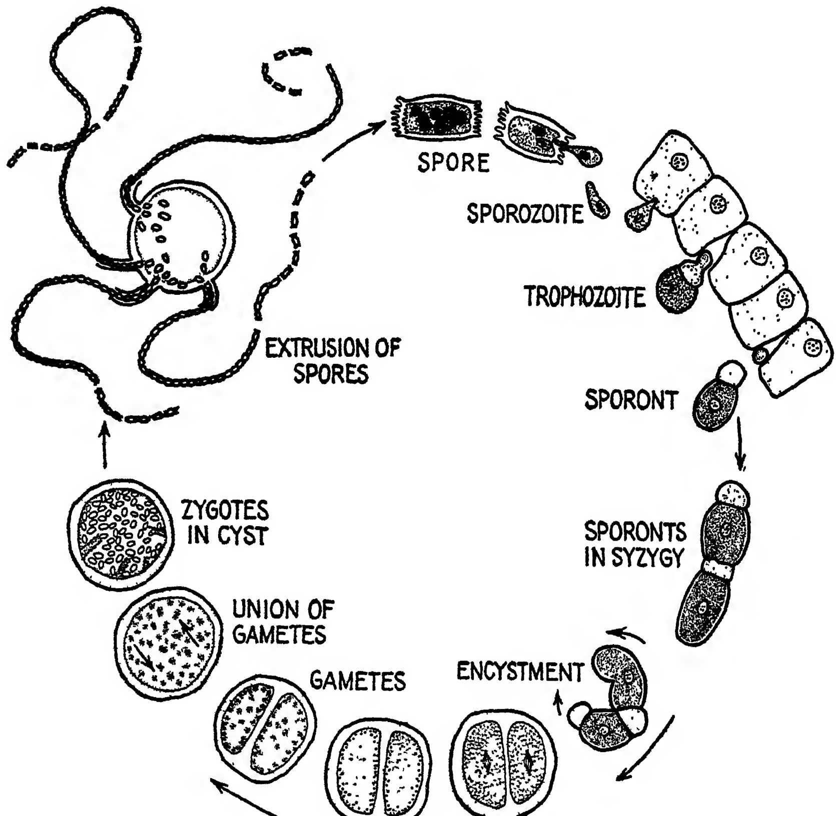


Fig. 176. Diagrammatic representation of the life cycle of a cephaline gregarine. (*In part after Watson, 1916; drawn by K. Snyder.*)

sporadin in an association known as "syzygy" in which the anterior gregarine is known as the "primite" and the posterior individual as the "satellite." Sometimes there may be several satellites attached one to the other. This tendency to associate is a characteristic for which the name *Gregarina* (Latin, *gregarius*) is derived. In some genera the sporadins are solitary during most of their existence and do not become associated until just prior to cyst formation.

Encystment begins when the paired sporadins rotate about a common axis and form a sphere that acquires a relatively thick covering. This

cyst soon leaves the body of the host along with the feces and, unless it falls into an unsuitable environment, continues its development. The nucleus of each of the two individuals (gametocytes) within the cyst divides repeatedly until a large number of small nuclei or chromidial bodies are formed, each acquiring a small amount of the sporadin's residual protoplasm. By a process of budding, these small nucleated particles transform themselves into numerous gametes that may be isogamous or anisogamous (*i.e.*, morphologically identical or morphologically dissimilar). The gametes of the two sporadins then intermingle with each other, join in pairs and unite, forming a large number of zygotes. Each zygote becomes surrounded by a transparent resistant membrane, and formation of the spore begins. The contents of the latter break up into a number of parts, usually eight, each with a portion of the zygote nucleus, and each of these parts develops into a sporozoite. In certain instances the spores may be liberated from the cyst through special spore ducts, or they may be liberated more directly. In any case they become scattered by the wind and rain over the foliage, grass, and ground. Eventually they may be ingested fortuitously by a suitable host along with its food. Once within the host's alimentary canal the spores germinate, the sporozoites are set free, and the life cycle is repeated: sporozoite → trophozoite → sporadin → gamete → zygote → spore → sporozoite.

Morphology. The Eugregarinina are usually divided into two more or less distinct groups or tribes. The members of one of these groups, Acephalina, have a body structure consisting of a single compartment. The body of those in the second group, Cephalina, consists of two compartments separated by an ectoplasmic septum. In the case of the latter tribe, the smaller anterior part is known as the "protomerite." The larger posterior part is the deutomerite, and it is this portion that usually contains the nucleus. Extending from the protomerite may be a structure, called the "epimerite," possessing hooks or other processes at its anterior border that serve as a means by which the organism may attach itself to the cells of its host. This structure is usually discarded after the gregarine has detached itself from the gut wall. Trophozoites that bear epimerites are termed "cephalines" (cephalonts).

The full-grown trophozoite is usually an elongated wormlike organism and in different species may vary from 10 to 16,000 microns (16 millimeters) in length. It is more or less constant in form, usually being corpulent and doliform to vermiform in shape. As seen free in the host gut, the sporadin moves about in various amoeboid, gliding, or wormlike fashions. The body of the gregarine is typically differentiated into an ectoplasm and endoplasm. The ectoplasm may consist of three layers: an external cuticle (epicyte), a middle layer (sarococyte), and a deeper contractile

layer (myocyte) containing muscular fibrils or myonemes. From the epicyte is derived the epimerite with its attaching processes and hooks. When septa are present these are derived from the sarcocyte. The endoplasm is usually granular and may contain a variety of inclusions, granules, spherules, and other bodies. Some of these represent glycogen spherules and other food materials stored up in reserve for the reproductive processes.

The typically single, vesicular nucleus of a gregarine is usually of a

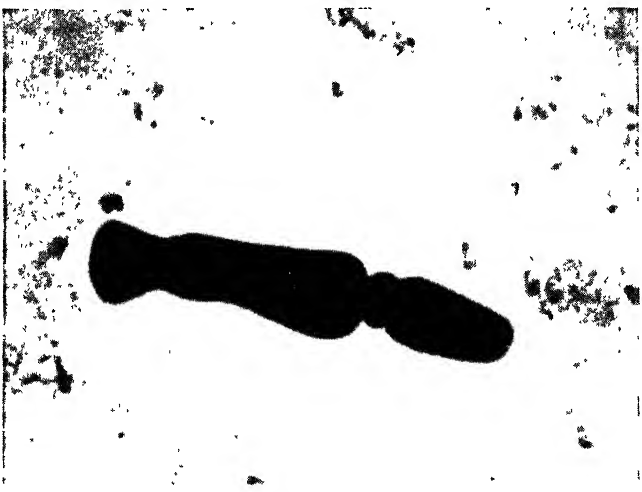


Fig. 177. *Gregarina cuneata* Stein from the gut of the yellow mealworm, *Tenebrio molitor* Linn. Photograph shows pair in syzygy. (*Photograph by K. M. Hughes.*)

large size and spherical or sometimes slightly elliptical in shape. One or more distinct karyosomes are usually present. (See also Allegre, 1948.)

Acephalina. In the suborder Eugregarinina the trophozoites may consist of but a single compartment, in which case they belong to the tribe Acephalina. The division of this tribe into a number of families is done on the basis of spore and sporadin characteristics. As compared with the cephaline gregarines, very few Acephalina have been found in insects, not more than 10 species having been described from these arthropods. For the most part, these have been placed in the families Monocystidae, Diplocystidae, and Allantocystidae.

One of the best known Acephalina associated with insects is *Lankesteria culicis* (Ross) found in the gut and Malpighian tubes of the mosquitoes *Aëdes aegypti* (Linn.) and *Aëdes albopictus* (Skuse). That this protozoan actually belongs to the tribe Acephalina has been questioned by Ray (1933), who maintains that it possesses a rudimentary protomerite and

therefore should be placed among the septate or cephaline gregarines. Recent writings of systematic protozoologists (*e.g.*, Kudo, 1946), however, retain the gregarine among the Acephalina.

Lankesteria culicis has been found in nearly all parts of the world where its mosquito hosts live. It was first observed in India by Ross (1895), who at that time referred to it as *Gregarina culicidis*. Its life cycle was elucidated by Wenyon (1911). The mosquito larva becomes infected when it ingests the oöcysts, or spores, deposited in the water by the adult mosquitoes. In the digestive tract of the larva the spore germinates, liberating eight sporozoites that enter the epithelial cells of the stomach where each becomes a spherical intracellular parasite. In this location it grows until it protrudes from the cell, though remaining attached by means of its epimerite. During this time the host cell has been largely destroyed. Eventually the parasite drops into the lumen of the gut where it moves about with characteristic movements. The fully grown gregarine is often about 50 microns in length, although some specimens exceed this dimension.

When the mosquito larva becomes a pupa, it no longer takes food, and the gregarines migrate from the gut to the Malpighian tubes. Here the parasites associate in pairs, each pair becoming enclosed in a spherical gametocyst within which the nucleus of each gregarine (gametocyte) divides, forming daughter nuclei. These daughter nuclei undergo repeated mitotic divisions which take place simultaneously in each of the paired gregarines. At the cessation of this division process, each gametocyte contains several hundreds of nuclei that pass to the periphery where they form small protoplasmic buds, each containing one nucleus. Each of these buds then separates off and becomes a gamete. The gametes having large nuclei are the female gametes; those having small nuclei are the male gametes. The male and female gametes conjugate in pairs to form a slightly elongated zygote that develops a cyst wall (oöcyst). Within each oöcyst eight sporozoites are formed, lying around a central mass of residual cytoplasm. Soon after the insect emerges from the pupal case, the gametocyst ruptures, and the oöcysts, or spores, are liberated into the lumen of the Malpighian tubes and then into the intestine of the mosquito. From here they are ejected into the water along with the insect's feces. The life cycle of the parasite is then repeated when the oöcysts, or spores, are ingested by mosquito larvae living in this water.

Cephalina. Most of the true gregarines associated with insects belong to the tribe Cephalina, those gregarines divided into two compartments by a septum and, hence, sometimes known as "polycistid gregarines." Most of the cephaline gregarines inhabit the alimentary canals of arthropods. Those associated with insects are, for the most part, found in the

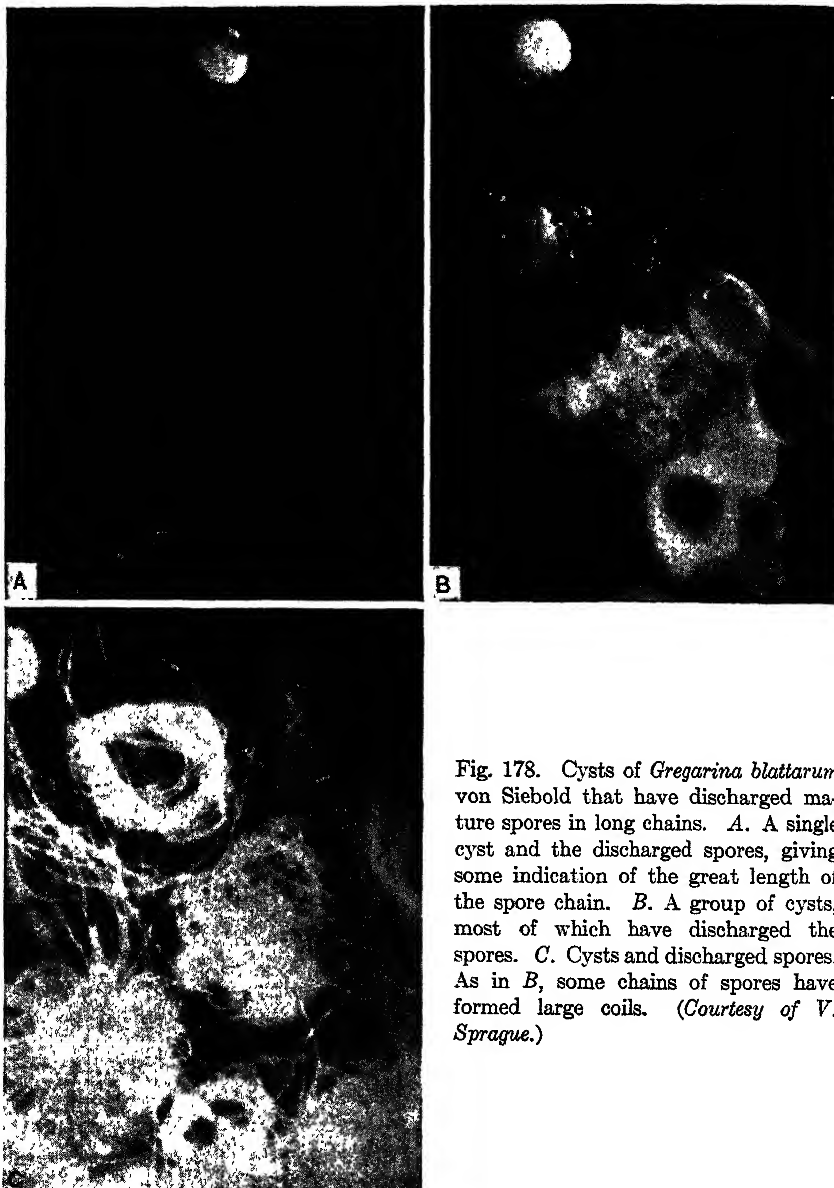


Fig. 178. Cysts of *Gregarina blattarum* von Siebold that have discharged mature spores in long chains. *A*. A single cyst and the discharged spores, giving some indication of the great length of the spore chain. *B*. A group of cysts, most of which have discharged the spores. *C*. Cysts and discharged spores. As in *B*, some chains of spores have formed large coils. (*Courtesy of V. Sprague.*)

numerous genera of several families.[1] Perhaps the best known of these are those belonging to the family Gregarinidae of which the type genus is *Gregarina*.

Typical of the cephaline gregarines is *Gregarina blattarum* described by von Siebold in 1839. This gregarine inhabits the alimentary tracts of the cockroaches *Blatta orientalis* Linn., *Blattella germanica* (Linn.), *Periplaneta americana* (Linn.), and *Parcoblatta pennsylvanica* (DeG.). Similar to *Gregarina blattarum* is *Gregarina cuneata* Stein found in the mealworm

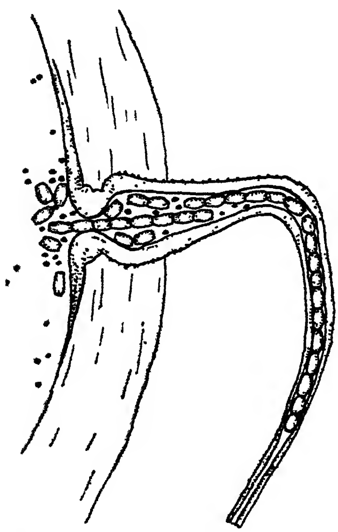


Fig. 179. Optical section of a sporoduct from a cyst of *Gregarina blattarum* von Siebold, showing manner of spore expulsion. (*Redrawn from Sprague, 1941.*)

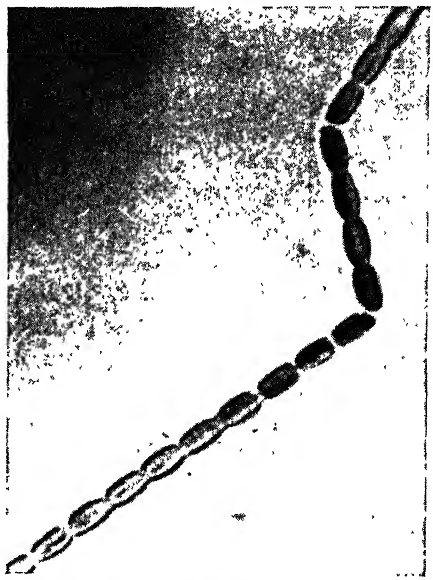


Fig. 180. Photomicrograph of a portion of a spore chain of *Gregarina blattarum* von Siebold. Enlarged to show individual spores. From life. (*Courtesy of V. Sprague.*)

Tenebrio molitor Linn. Both these gregarines have well-developed epimerites by which they attach themselves to the epithelial cells of the host's gut. The sporadins of both species characteristically may associate in pairs soon after the loss of their epimerites. The life cycle of these gregarines is essentially the same as the general one described on an earlier page. A few additional words, however, may be given on the interesting reproductive phases that occur in this type of gregarine.

The encystment process for *Gregarina blattarum* von Siebold has been

[1] Lecudinidae, Didymorphyidae, Gregarinidae, Leidyanidae, Monoductidae, Menosporidae, Dactylophoridae, Stylocephalidae, Acanthosporidae, and Actinocephalidae.

studied by several investigators including Sprague (1941), who observed the process after pairs of gamonts (the initial stage in gamete formation) had been placed in fresh undiluted egg albumen on a depression slide. Imminent encystment, which seldom occurs until the gamonts have reached a certain definite minimum size, is indicated by characteristic rotating movements of the paired gregarines. The two individuals glide slowly forward, the anterior end of one bending in a direction so as to meet the bending posterior end of the other, so that there is a tendency to move in a circle. After the two ends are finally brought together, they adhere and the rotation of the pair continues. Before long a cyst membrane is secreted which encloses the gamonts which then proceed, in the usual fashion, to the eventual production of gametes and then spores.

The spores are liberated from the cyst through special structures known as "sporoducts." The sporoduct in *Gregarina blattarum* is a tapering tube about 200 microns long with a bulbous enlargement near its base (Fig. 179). As a cyst approaches maturity, the basal disc of the sporoduct becomes slightly raised, forming a small convex protuberance. At this point the cyst membrane is slightly thinner than elsewhere, and the sporoduct bursts through this area, which Sprague believes may have been weakened by the action of an enzyme. In bursting through the cyst membrane the sporoduct turns inside out and becomes completely extended. First to emerge from the sporoduct are a few oil droplets, which possibly lubricate the sporoduct walls for the rapid passage of spores which are liberated a moment later. The spores are discharged in long chains, which frequently become arranged in large coils. Some chains have been observed to contain as many as 10,000 spores held together by an adhesive mucoid sheath. The size of the individual spores average 8.5 microns long by 4.0 microns wide.

Schizogregarinina

On a foregoing page it was explained that the members of the suborder Schizogregarinina, a smaller group than Eugregarinina, undergo both sporogony and schizogony. Sporogony is of the type already described for the eugregarines, and schizogony may take place by binary fission, multiple fission, or budding. Schizogony may occur either outside or within the host cell.

Representatives of both schizogregarine families (Ophryocystidae and Schizocystidae) are found associated with insects as well as with annelids and tunicates. There have, however, been scarcely more than 25 species of schizogregarines described from insects. Most of these have been found in Coleoptera, Diptera, and Hemiptera.

Ophryocystidae. In the gregarines of this family one spore is formed

from two gametocytes, which fact differentiates it from the Schizocystidae. *Ophryocystis* is the principal genus in the family, and it is known to contain about 10 species that occur in the Malpighian tubes of Coleoptera. *Ophryocystis mesnili* Léger, is found in the Malpighian tubes of the yellow mealworm, *Tenebrio molitor* Linn., and *Ophryocystis hessei* Léger is parasitic in the beetle *Omophlus brevicollis* Muls. After being ingested by a suitable host, each of the spores of these gregarines liberates eight sporozoites which soon make their way to the Malpighian tubes where they attach themselves to the surface of the cells. Here each parasite grows and becomes a multinucleate adult, which then segments into a number of merozoites. These merozoites, in turn, attach themselves to the cells and grow into adults. After this is repeated several times schizonts containing several nuclei are produced. After segmenting, these forms come together as paired gametocytes, as in the case of the eugregarines. Three nuclei are produced within each of the encysted gametocytes. One of these nuclei becomes the nucleus of the gamete, and thus one gamete is produced by each of the two gametocysts, making two solitary gametes in the gametocyst. These two gametes conjugate and are encysted in a spindle-shaped spore or oöcyst within which are formed eight sporozoites.

In 1948 Ghélélovitch proposed the genus *Coelogregarina* to include *C. ephestiae* Ghél., a parasite of the Mediterranean flour moth, *Ephestia kuhniella* Zeller. The spores of this schizogregarine germinate in the intestinal tract of the host, and the sporozoites infect the cells of the fat body after having penetrated the intestinal wall. The mortality caused by the parasite is great in the larvae but only minor in the adult insects. Ghélélovitch found one larva to be parasitized simultaneously by *Coelogregarina* and by a microsporidian and a coccidian. Larvae of the lesser wax moth, *Achroia grisella* Fabr., and the wax moth, *Galleria mellonella* Linn., are also susceptible to *Coelogregarina ephestiae* Ghél. Toumanoff (1948) suspects that *Dibrachys boucheanus* Ratz., a calcid parasite of the lesser-wax-moth larva, is capable of transmitting the protozoan in cultures of this insect.

Schizocystidae. In the members of this family the union of two gametocytes produces two or more spores instead of only one as in the Ophryocystidae. Species have so far been described from Diptera and Hemiptera.

Representative of this family is *Schizocystis gregarinoides* described by Léger (1900) as a parasite of larvae of the midge *Ceratopogon solstitialis* Winn. After being ingested by this insect, the oöcysts release sporozoites which commence the infection. After attaching itself to the gut epithelium of the insect, each sporozoite develops into an elongated vermiform body, which may reach the length of 150 microns. The nucleus multiplies and by schizogony produces a number of merozoites which again attach

themselves to the gut wall and become schizonts. After this process repeats itself several times, the merozoites develop into gametocytes

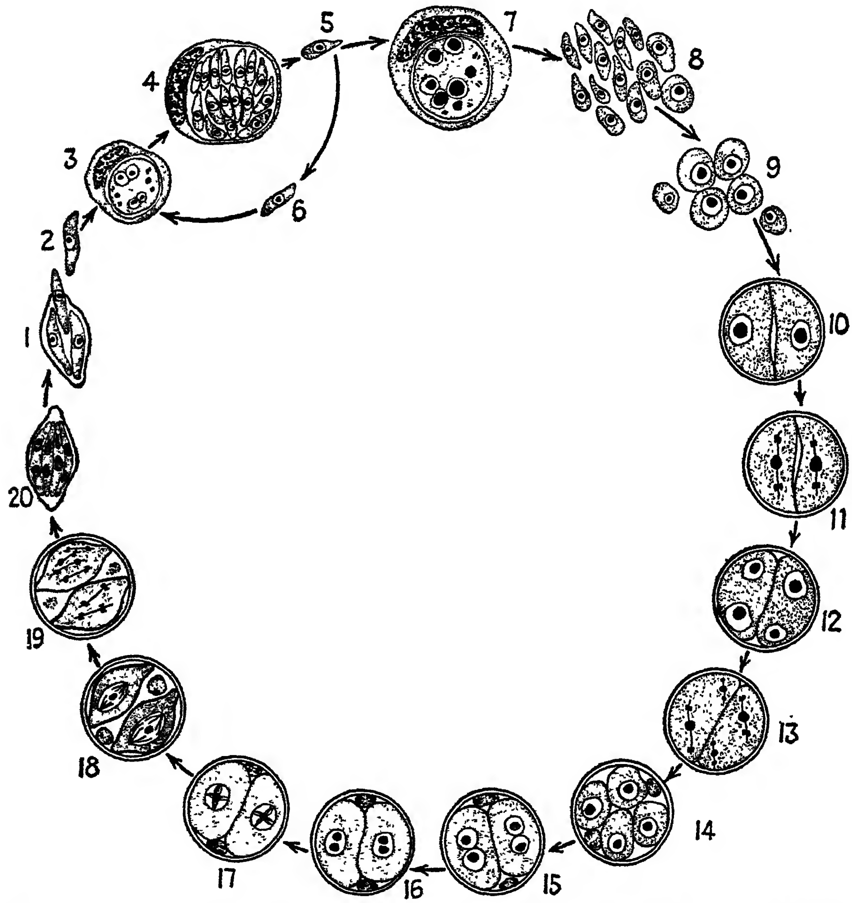


Fig. 181. A schematic representation of the life cycle of the schizogregarine *Mattesia dispora* Naville. 1. A germinating spore with the emerged sporozoite (2) which penetrates an adipose cell (3) of the host insect (the larva of the Mediterranean flour moth, *Ephestia kühniella* Zeller). 3–8. Schizogony. 8 and 9. Gamonts. The gamonts associate in pairs around each pair of which a cyst is formed. 10–14. Gamete formation. 15. Zygote formation, each zygote developing (15–19) into a spore as shown in 20. (*Redrawn from Naville, 1930.*)

which become paired and form gametocysts, in much the same fashion as do the eugregarines. A number of gametes are formed in each gametocyst. After conjugating in pairs, the gametes form a number of spindle-shaped oöcysts, each containing eight sporozoites. The oöcysts are eliminated

with the insect's feces; and, if they are taken up again in the food of a host, the cycle is repeated.

Other genera of Schizocystidae containing entomogenous members are *Snycystis*, *Mattesia*, *Caulleryelle*, *Lipotropha*, and *Machadoella*. The different species of these genera show many interesting variations in the relations to their hosts. Some are well-tolerated parasites; others are rather destructive to the host insect. Of the latter group, one of the most interesting species is *Mattesia dispora* Naville, which has been found (Naville, 1930; Musgrave and Mackinnon, 1938) pathogenic for larvae of at least two Lepidoptera, *Ephestia kühniella* Zeller and *Plodia interpunctella* (Hbn.). Complete or partial destruction of cultures of these insects took place in European laboratories. What apparently is the same protozoan has been found in cultures of these insects in the United States. Schizogony takes place in the fat cells of the host. Each pair of gametocytes produces two spores. The life cycle of this parasite is shown in Fig. 181.

General Biological Aspects

Insect Hosts. Gregarines are found only in invertebrates, especially in arthropods, annelids, tunicates, and mollusks. In most of these animals the gregarines are parasitic in the alimentary tract. The majority of entomogenous gregarines so far described are from Coleoptera, with Orthoptera and Diptera being next in line as to frequency of hosts. A hasty perusal of the literature has yielded the following tabulation of the number of different species of insects in each order which have been found as hosts to gregarines:

Coleoptera......	180	Lepidoptera	3
Orthoptera......	67	Isoptera.........	3
Diptera.........	35	Hymenoptera	2
Trichoptera.....	12	Plecoptera.......	2
Odonata........	8	Neuroptera......	1
Thysanura......	6	Collembola.......	1
Ephemerida. ...	3	Embioptera.. ...	1
Hemiptera... .	3		

This list is not complete, but it probably represents the relative proportions of gregarines found in the various insect orders. It is interesting that the members of such orders as Siphonaptera, Corrodentia, and Mallophaga are rarely, if ever, found to harbor gregarines.

Anatomical Location of Gregarines. In most cases the gregarine sporadins are located in the midgut of the host. They are found in the esophagous, crop, and rectum only when the infection is extremely heavy. Occasionally the pyloric caeca are infected, and in a few insects the para-

sites have been found in the coelom and attached to the intestinal wall. The sporadins may lie loosely among the contents of the lumen of the gut. In most cases, however, the parasites lie rather close to the epithelial walls and are not scattered through the food masses. The cysts of gregarines are sometimes found in the midgut of the insect host, but usually they are recovered from the rectum.

As was pointed out in an earlier paragraph, the infecting sporozoites and the developing trophozoites may live entirely within the epithelial cells of the host's intestine during the early part of their existence. Eventually they emerge from the impaired or destroyed cell, remaining attached a while by their epimerites, or they are released freely into the intestinal lumen.

Numbers of Gregarines. The number of gregarines in any particular host species may vary considerably, depending upon several factors. In the eugregarines asexual reproduction does not occur; and, once a gregarine matures and forms a cyst, it must await its reintroduction into another host before the numerous sporozoites are freed from the spores, enabling them to attack new host cells. Since there is no actual multiplication within a single host, the latter does not become intensely infected, as is the case with certain other protozoan parasites. The Schizogregarinina, on the other hand, multiply asexually as well as sexually, and hence the infection in any single host may reach a relatively greater degree than is the case with Eugregarinina.

The numbers of gregarines in a particular species of insect may also vary according to the season of the year. Frequently the insect concerned is found to harbor a considerably greater number of gregarines in the fall of the year than it does in the earlier seasons; *i.e.*, instead of an infected insect harboring from 1 to 10 parasites as in the spring of the year, it may be found to contain from 50 to 100 or more in the fall. Furthermore a greater number of insects are infected in the fall than earlier in the year. Particularly are these facts true wherever the ecological niche is fairly well defined and more or less restricted. Workers rearing insects in cages have frequently found the gregarine infection to increase as time went on both as to the number of insects infected and as to the number of gregarines present in each individual host. This situation is brought about by the frequency of contact between hosts and their droppings and by the increased density of spores in the environment.

Effect of Parasite on Host. The relation of gregarine parasites to the tissues of their host is not at all clear. We have already pointed out that, although most authorities consider gregarines to be parasites, it has not been shown that they are especially harmful to their hosts. That they may have a general debilitating effect upon their host is possible.

Very few histopathological data are at hand. It is known, however, that after the sporozoite has penetrated the host cell and come to rest in the vicinity of the nucleus, the latter is markedly affected. The chromatin of the nucleus soon begins to break up and rearranges itself into small more or less spherical bodies that have tinctorial properties different from those of the normal nucleus. That the cytoplasm is also affected is indicated by the fact that it stains less deeply than does the cytoplasm of a normal cell. In some cases the affected epithelial cell has been seen to hypertrophy to a size ten or more times that of a normal cell. In any case the growing parasite eventually breaks out and the affected cell shrinks, disintegrates, and disappears, the adjoining cells gradually filling in the vacated space. Thus we are concerned primarily with a pathology of individual cells and perhaps of a few surrounding cells, the destruction of which does not in itself greatly affect the host as long as the infection is a relatively mild one. In some instances, however, the destruction may be more generalized, as in infections with *Mattesia dispora*, which has definite pathogenic properties.

Now, as to the nature of the underlying cause of this cellular destruction, there has been some theorizing but very little experimental proving. Some of the damage undoubtedly is mechanical in nature, the mere growth and increased size of the parasite being sufficient to disrupt the cell's metabolic processes. The direct utilization of the cytoplasmic material by the parasite for its growth shares in the destruction. The disastrous effect of the parasite on the host cell has been thought to be caused by a chemical substance secreted by the gregarine soon after its penetration into the cell. It has been postulated that this substance first stimulates the growth of the cell but later retards it and kills the cell. Whether this substance is in the nature of a distinct secretion of merely normal excretory products is not clear. No direct evidence of a special secretion or toxin is as yet available. If the product were excretory in nature, it would seem that the host cell would have to remain alive to eliminate the substance or else, as is generally the rule with animals, the gregarine would be poisoned by its own excretory products. Some authors (*e.g.*, Watson, 1916) have therefore questioned whether the host cell is killed by the entrance of this foreign substance rather than being killed gradually by the utilization of the cell's protoplasm as food.

It appears that gregarines are also capable of deriving nutrient materials from the host cell while they are attached to the latter by their epimerites through which absorption may take place. During the greater part of the cephaline life of the parasite probably very little nourishment is absorbed through its outer covering or epicyte (Watson, 1916).

That gregarines are actually essential for the normal growth of some

insects has been claimed but not definitely confirmed. Sumner (1936), for example, reported that larvae of the mealworm, *Tenebrio molitor* Linn., harboring gregarines (*Gregarina steini* Bern.), grew more rapidly and had a lower mortality rate than did gregarine-free larvae. It remains to be seen whether this apparent beneficial effect is brought about by the gregarines themselves or by other associated factors. Earlier, Portier (1919), after growing the mealworm on sterile media, concluded that microorganisms were not essential for the growth of this insect.

COCCIDIAN INFECTIONS IN INSECTS

The order Coccidia belongs to the same subclass (Telosporidia) of Sporozoa as do the gregarines. They differ, however, in numerous respects, one of the principal differences being that the mature trophozoite of the Gregarinida is large and extracellular while that of the Coccidia is small and intracellular. The Coccidia may be differentiated from the Haemosporidia by the fact that in the latter the zygote is motile and the sporozoites are naked and not enveloped.

The Coccidia are found parasitizing many species of vertebrates and invertebrates in which they attack the epithelium of the digestive tract and its associated glands and frequently other internal tissues. Reproduction occurs both asexually by schizogony and sexually by anisogamy (*i.e.*, male and female gametes morphologically unlike), in most cases both types of reproduction taking place in the same host body.

Depending upon whether the gametocytes are similar or dissimilar, and upon other characteristics, the Coccidia may be separated into two suborders: Eimeridea and Adeleidea. Most of the species parasitizing insects fall in the suborder Adeleidea, family Adeleidae. There are reports of Eimeridea in insects, however, such as *Barrouxia ornata* Schneider in the gut of the hemipteran *Nepa cinerea* Linn.

Adeleidae

Adelina. The genus *Adelina* is one of the most important of the Coccidia from the standpoint of causing infection in insects. It was established in 1911 by Hesse for a coccidian that he described from the oligochete *Slavinia appendiculata*. He named the parasite *Adelina ocotospora* and separated it from the members of the genus *Adelea* on the basis of the spherical shape of the sporocysts which in *Adelea* are discoidal. Upon making this separation it therefore became necessary to transfer several earlier described species from the genus *Adelea* to the genus *Adelina*. At present the genus is known to contain the following species that parasitize insects:

Adelina simplex (Schneider, 1885) in *Gyrinus* larvae
Adelina tipulae (Léger, 1897) in *Tipula* larvae
Adelina mesnili (Pérez, 1899) in *Tineola bisselliella* (Hum.); and probably in *Ephestia kühniella* Zeller, and *Plodia interpunctella* (Hbn.) (Steinhaus, 1947)
Adelina akidum (Léger, 1900) in *Olocrates abbreviatus* Muls.
Adelina transita (Léger, 1904) in *Embia solirei* Ramb.
Adelina zonula (Moroff, 1906) in *Blaps mortisage* (auctt., Linn.)
Adelina tenebrionis Sautet (1930a) in *Tenebrio molitor* Linn.
Adelina tribolii Bhatia (1937) in *Tribolium castaneum* (Hbst.) (*T. ferrungineum* (Fabr.))
Adelina cryptocerci Yarwood (1937) in *Cryptocercus punctulatus* Scudd.

Life Cycle of *Adelina*. The life cycles of the various species of *Adelina* are essentially the same. To serve as an example, the life cycle of *Adelina cryptocerci* as described by Yarwood (1937) in the wood-eating roach *Cryptocercus punctulatus* Scudd. may be recounted here. About 10 hours after ripe oöcysts are ingested by the roach, some of the oöcyst walls have opened and numerous free sporocysts may be seen in the lumen of the gut; after 24 hours, free sporozoites may be seen. Their average size is 2 to 3 by 15 to 17 microns. The sporozoites pass through the wall of the midgut into the hemolymph surrounding the intestine. From here they go to the fat bodies and other tissues of the insect where they increase in size to form schizonts. The first schizogony, in which merozoites are produced, takes place in the fat bodies between the sixteenth and twenty-fifth day after ingestion of the cysts. The merozoites first occur in barrel-like bundles of up to 40 individuals. After freeing themselves from this arrangement, the merozoites are found in various positions and shapes throughout the fatty tissues. According to Yarwood, these merozoites give rise to a second schizogonial generation in which two types of schizogony produce merozoites and gametoblasts, respectively. This observation has not been confirmed and is not considered typical of all *Adelina*. Furthermore, that male and female gametocytes can arise from the nucleus of one and the same merozoite, as reported, has been questioned (Hauschka, 1943). Some workers (*e.g.*, Pérez, 1903, in the case of *Adelina mesnili*) believe that the microgametocytes arise from the thick short merozoites while the macrogametocytes develop from the more elongated fusiform merozoites.

At any rate the parasite passes from the sporozoite stage to the merozoite stage from which are derived the small male (micro-) and large female (macro-) gametocytes or, as designated by Yarwood, gametoblasts. Male and female gametocytes become associated during the growth period of the latter. One or two microgametocytes become attached to the macrogametocyte. As the latter attains full size and becomes rounded the microgametocyte moves to one end, rounds out, and becomes flattened

on the female. They then become surrounded by a membrane (gametocyst). In the roach this stage is usually reached in about 29 days after ingestion of the oöcysts.

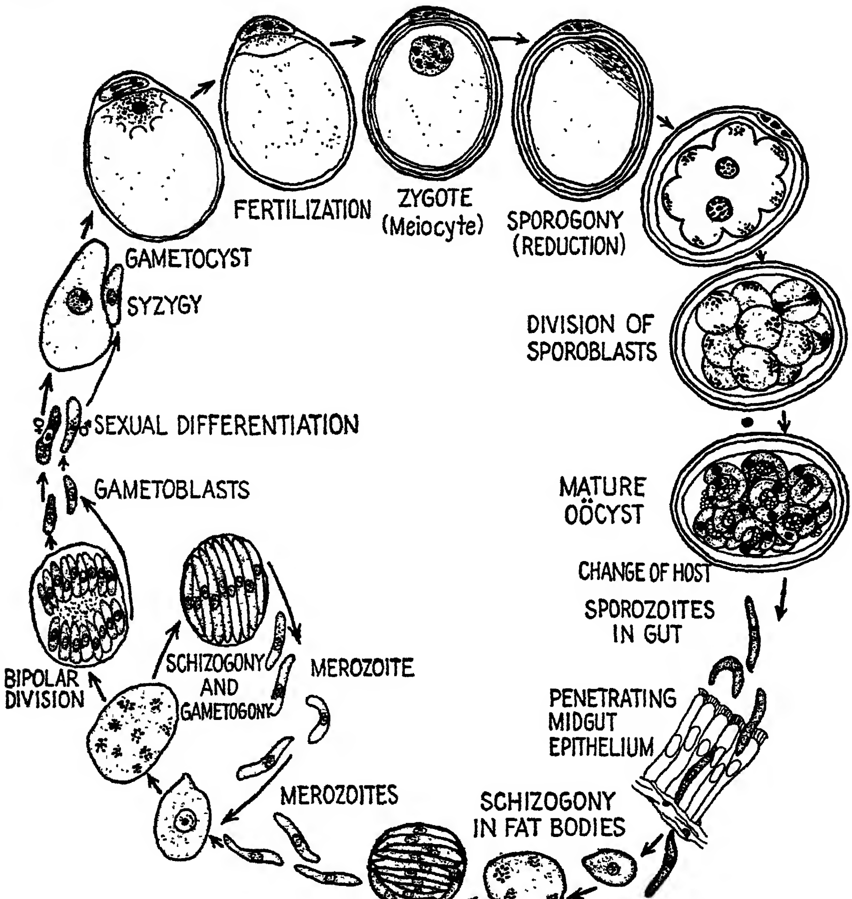


Fig. 182. Diagrammatic representation of the life cycle of *Adelina cryptocerci* Yarwood, a coccidian parasite of the wood-eating roach *Cryptocercus punctulatus* Scudd. (*Redrawn from Yarwood, 1937.*)

The macrogametocyte nucleus moves from its position near the center toward one end, where it comes to lie close to the surface. The nucleus of the microgametocyte divides twice, forming four microgamete nuclei, which become condensed into darkly staining comma-shaped structures. One of the four microgametes passes through the surface membrane of the macrogametocyte, now called the "macrogamete," and fuses with its nucleus. Within a short time this newly formed zygote is surrounded by

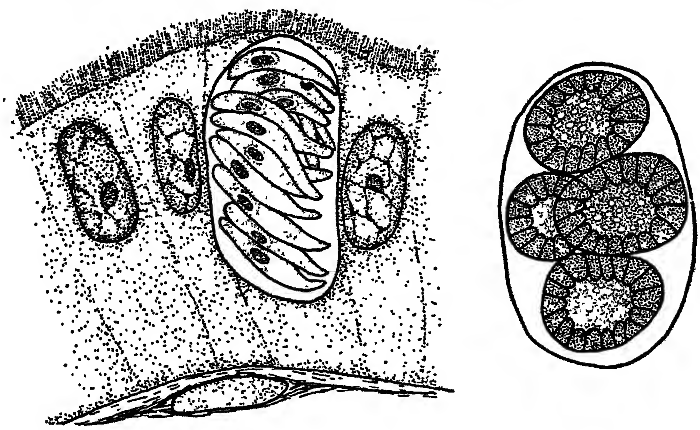
a membrane that encloses the three unused microgametes between itself and the gametocyst. Subsequent to this, a third membrane is formed making a cyst wall of three-layer thickness. The zygote nucleus undergoes repeated divisions and forms a sporont with from 5 to 21 nuclei. About each of these nuclei is formed a sporoblast. The nucleus of each sporoblast divides once. A cyst wall is formed about each sporoblast, thus forming sporocysts. Each of the two nuclei and their accompanying cytoplasm go to form the two sporozoites that lodge in each sporocyst. The entire structure containing the sporocysts varies from 10 to 12 microns in diameter. The size of the oöcyst varies with the number of sporocysts it contains. In the case of *Adelina cryptocerci*, Yarwood (1937) found that the size of the oöcyst varied from one 24 by 28 microns containing 5 sporocysts to one 46 by 51 microns containing 21 sporocysts. The average size was about 31 by 41 microns, and the average number of sporocysts in 31 oöcysts was 10.4. The entire life cycle from the time the original ripe oöcysts were ingested takes approximately 40 days, although this varies somewhat with the species of *Adelina* concerned.

Effect of *Adelina* on Host. The detrimental effect of a coccidian upon its insect host to some extent depends upon the intensity of the infection. In advanced heavy infections the parasites may be present in nearly every part of the body. In early light infections the protozoa may be confined to the fat bodies in the area about the alimentary tract. In the latter case, very few visible effects are noticeable in a single insect specimen. In the more advanced infections there are usually definite symptoms discernible upon close and careful examination of the host.

Heavily infected larvae are sluggish and slow in movement. Reaction to external stimuli is markedly reduced. The color of the larvae may be modified or slightly abnormal. Infected adults may also be sluggish in movement and somewhat slow to respond to stimuli. There is evidence that the reproductive capacity of infected insects is reduced. Infected colonies slowly lose their vitality, decrease in numbers, and eventually may die out altogether.

Although insects may show the ill effects of a coccidian infection, it is nevertheless remarkable how they withstand even heavy parasitization. Insects infected as larvae may continue their development and metamorphosis through to mature adults and, in general, maintain all their principal habits and functions. Even lepidopterous insects undergoing complete metamorphosis carry the coccidia with them through the pupal stage to the adult insect. Because of the general tolerance that insects have toward these parasites, it is doubtful if the latter would ever prove very effective as a means of large-scale rapid control of susceptible insects except where conditions were such that the debilitating effect of the

parasite was great enough to be of deciding importance. It is important, however, to avoid coccidian infections in insectaries and other places where insects are being reared. Not only may the insect colony eventually be lost, but it would consist of anything but normal insects and could not be used for most experimental purposes. Preventive measures include the strict adherence to rules of sanitation and cleanliness. When possible, sterilization of rearing jars and cages by steam or boiling water should be used to destroy the resistant stages of the parasite.



A B

Fig. 183. A coccidian, *Ithania wenrichi* Ludwig, in the cranefly, *Tipula abdominalis* Say. *A*. Mature schizont in the midgut epithelium of host larva. *B*. Oöcyst containing four spores. (*Redrawn from Ludwig, 1947.*)

Just how the protozoan gets from the coelom of one insect to the gut of another is not known with certainty. Cannibalistic tendencies of some insects may be instrumental in the transmission, but probably a greater number of oöcysts are transferred after the infected host dies and its tissues disintegrate and mingle with the soil or other environment of the insect so that it is capable of being picked up or ingested by another susceptible insect.

Other Adeleidae. Other Adeleidae parasitic in insects are included in the genera *Legerella, Chagasella,* and *Ithania.*

The first species of *Legerella* recorded from insects was *Legerella parva* found by Nöller in 1914 in the Malpighian tubes of the chicken flea, *Ceratophyllus gallinae* (Shrank), and the pigeon flea, *Ceratophyllus columbae* Walk. & Ger. A few years later *Legerella grassii* Splend. was found parasitizing the Malpighian tubes of *Nosopsyllus fasciatus* (Bosc d'Antic). In 1927, Vincent reported on *Legerella hydropori*, a parasite of *Hydroporus palustris* Linn., a small dytiscid beetle common in stagnant ditches in England. The infection is confined to the Malpighian tubes of the insect

and may be very intense. Because the Malpighian tubes are colorless and do not contain the brown excretory granules characteristic of normal tubes, Vincent was able to distinguish with ease the heavily infected beetles at the time they were dissected. The parasite may be present in the insect at all seasons of the year, and the life cycle apparently does not follow a seasonal course. In heavily infected beetles, the epithelial cells of the Malpighian tubes may contain numerous schizonts. The cells may be considerably enlarged, and, where active schizogony is taking place, the destructive effect on the epithelium is usually considerable. The host nuclei proliferate in the presence of the parasite and may occasionally be seen undergoing mitotic division.

Chagasella hartmanni (Chagas) is a coccidian parasite of the hemipteran *Dysdercus ruficollis* (Linn.). It develops in the epithelial cells of the intestine of this insect. Another closely related species, *Chagasella alydi* Mach., is parasitic not only in the intestine but also in the reproductive organs of its host (*Alydus* sp.).

Ithania wenrichi Ludwig parasitizes the epithelial tissue of the caeca and midgut of the larvae of the cranefly, *Tipula abdominalis* Say. Cranefly larvae collected from streams in Pennsylvania showed an average incidence of infection with this coccidian of about 70 per cent (Ludwig, 1947).

Haemogregarinidae. The family Haemogregarinidae includes those coccidia which have both a vertebrate and an invertebrate host. The protozoa live in the circulatory system of the vertebrate host and in the digestive system of the invertebrate host. In some instances the invertebrate host is an insect that usually is not adversely affected to any great extent, although definite tissue destruction may occur. An example of this dual host relationship is *Haemogregarina triatomae*, whose vertebrate host is a South American lizard (*Tupinambis teguixin*), and whose invertebrate host is the reduviid *Triatoma rubrovaria* (Blanch.).

The genus *Hepatozoon* has several members, the invertebrate hosts of which are arthropods. *Hepatozoon muris* (Balfour), for example, is found in common rats in many parts of the world. Its invertebrate host is the mite *Echinolaelaps echidninus* (Berlese) [= *Laelaps*]. The protozoan undergoes its schizogony cycle in the liver of the rat from where the young gametocytes invade the monocytes. When the infected blood is ingested by the mite, the parasites are liberated from the monocytes and associate in pairs. The union of the two gametes results in a vermiform organism that then penetrates the intestinal epithelium and goes through the intestinal wall to the surrounding tissues where it becomes spherical and grows. Eventually it becomes a cyst, the contents of which break up into sporoblasts and then into spores. Several sporozoites are contained in each spore. When the rat ingests the mite, the sporozoites are liberated

and infect the vertebrate host. The other species of *Hepatozoon* have similar host relationships.

Karyolysus lacertarum (Dan.), a parasite of a lizard (*Lacerta muralis* Boul.), undergoes its sexual reproduction in the female of the mite *Liponyssus saurarum* Oudemans. The parasitization of the mite is of a very interesting type. The protozoan sporokinetes enter the ova of the mite and here mature. During the tissue differentiation of the mite embryo the sporocysts occupy cells which eventually become the mite's gut epithelium. After the mite's first blood meal the spores are discharged from the body. The lizard becomes infected when it happens to ingest these spores.

HAEMOSPORIDIAN INFECTIONS IN INSECTS

The insect pathologist is usually concerned more with those microorganisms parasitizing or infecting insects directly. Occasionally, however, he must recognize the importance to his field of certain microorganisms that are known primarily because they are parasites of man, other animals, or plants and only incidentally because they are parasites of insects. In most of these cases the insect serves as a vector of the infecting agent. These microorganisms frequently cause important pathological changes in the insect, and the insect pathologist must be able to recognize these changes and distinguish them from those brought about by the primary pathogens of insects.

The members of the order Haemosporidia constitute just such a group of protozoa. The insect pathologist is rarely concerned about them or the infections they cause. He should, however, recognize the fact that they invade the tissues of their insect host and bring about certain histopathological changes.

The Haemosporidia undergo schizogony in the blood of vertebrates and sporozoite formation in the alimentary tracts of invertebrates. They always remain within the body of one or two hosts, and hence the sporozoites are without a protective envelope. The order is generally separated into three families: Plasmodiidae, Haemoproteidae, and Babesiidae.

The family Plasmodiidae includes the protozoa that cause malaria, not only of man but of other mammals, birds, and reptiles as well. These protozoa are transmitted by mosquitoes and their life cycles in these insects and in their vertebrate hosts are comparatively well known, at least for those forms infecting man.

Plasmodium vivax (G. & F.), the cause of tertian malaria in man, after being taken up by an anopheline mosquito (*e.g.*, *Anopheles quadrimaculatus* Say) from the blood of a human being, penetrates the stomach

wall of the insect and lodges between the inner and outer linings of the stomach. Here the parasite grows rapidly while a cyst wall develops, formed partly by the parasite and partly by the elastic membrane of the mosquito's stomach. If, at this point, the dissected stomach of an infected mosquito is examined, the cysts may be seen on the outer surface of the stomach protruding like tiny tumors or warts, 40 to 60 microns in diameter. The stomach tissue continues to function, but undoubtedly considerable strain is placed upon its cells in order to compensate for the considerable amount of destruction suffered as the result of the infection. When the cyst wall ruptures, the sporozoites within are released and make their way to the salivary glands where they remain until they are inoculated into the blood of a human being. Here again, the tissues of the salivary glands undergo considerable cellular alteration as the result of the invasion of the parasite. In spite of the great amount of work done on malaria parasites and their vectors, very little is known concerning the pathology caused by the parasite in the mosquito.

In the family Haemoproteidae schizogony takes place in the endothelial cells of the vertebrate host rather than in the peripheral blood, as in the case of Plasmodiidae. Birds and reptiles are the vertebrate hosts of the members of Haemoproteidae. Examples are *Haemoproteus columbae* C. & S. found in the pigeon *Columba livia* Gmelin and transmitted by certain flies, particularly those of the genus *Lynchia;* and *Leucocytozoon simondi* (M. & L.)(= *L. anatis* Wick.) which occurs in wild and domestic ducks and is transmitted by the black fly, *Simulium venustum* Say. The pathologies in the insects are practically unknown.

Members of the family Babesiidae are minute nonpigmented parasites of the red blood cells of mammals and are transmitted by ticks. *Babesia bigemina* (S. & K.), the cause of Texas fever or red-water fever of cattle, is transmitted by *Boophilus annulatus* (Say). The parasites may penetrate the cells lining the gut of the tick, grow, and form sporonts in this location, or they may pass completely through the gut wall, enter the ovary, and invade the ova. Some authors believe that the parasite migrates throughout the embryonic tissue cells of the tick, including the cells destined to develop into the salivary glands. Others workers have been unable to find any signs of invasion of the salivary glands. *Theileria parva* (Theiler), the agent of an important disease of cattle in Africa, is transmitted by *Rhipicephalus appendiculatus* Neum. and other ticks. This parasite enters the epithelial cells lining the gut of the tick, from where they make their way into the body cavity and eventually enter the cells of the salivary glands. The affected host cells are apparently destroyed or injured in this process.

MICROSPORIDIAN INFECTIONS IN INSECTS

The sporozoa we have so far been discussing belong to the subclass Telosporidia. We come now to the remaining two subclasses, Acnidosporidia and Cnidosporidia; the former having simple spores without that peculiar structure known as the "polar filament," the latter having resistant spores with polar filaments.

The Acnidosporidia are a very inadequately known group. They are usually divided into two orders: Sarcosporidia, which occur in the muscles

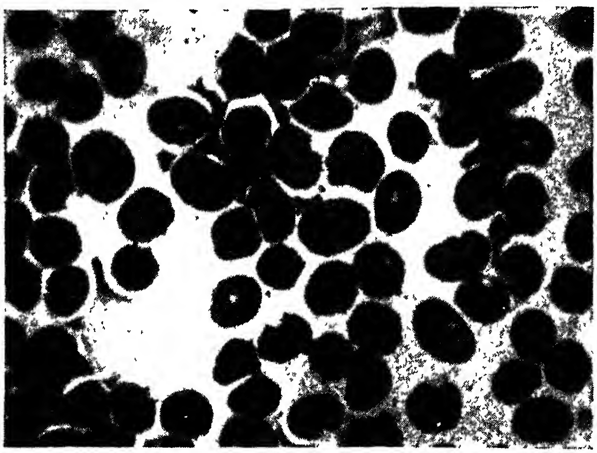


Fig. 184. Spores of *Haplosporidium ecdyonuris* Weiser from fat body of *Ecdyonurus venosus* Fabr. (*Courtesy of J. Weiser.*)

of higher vertebrates (and which some authorities now consider as belonging to the fungi rather than the protozoa), and Haplosporidia, found in fish and invertebrates. The latter group contains a few species known to infect insects. *Coelosporidium periplanetae* (L. & S.) and *Coelosporidium blattellae* Craw., for example, commonly occur in the lumen of the Malpighian tubes of cockroaches. *Nephridiophaga apis* parasitizes the honeybee, *Apis mellifera* Linn.; *Serumsporidium melusinae* Nöl. infects the dipteran, *Simulium reptans* Linn.; *Haplosporidium ecdyonuris* Weiser occurs in the fat body of *Ecdyonurus venosus* Fab.; and both *Haplosporidium bayeri* Weiser and *Coelomycidium ephemerae* Weiser parasitize *Cloeon rufulum*. These protozoa produce simple spores that superficially may resemble those of Microsporidia, except that they do not have polar filaments. In some species the spore has a lid through which the uninucleate sporoplasm emerges.

The Cnidosporidia may be separated into four orders: Myxosporidia, parasites of lower vertebrates, especially fish; Actinomyxidia, inhabitants

of the body cavity or the gut epithelium of fresh- or salt-water annelids; Microsporidia, parasites primarily of arthropods and fish; Helicosporidia, parasitic in arthropods. The last two orders, and particularly Microsporidia, are the only ones that concern us here. The microsporidian diseases (microsporidioses) of insects have had an early and important bearing on the development of the field of insect pathology generally and merit a detailed treatment in a book of this kind.

The order Microsporidia is usually divided into two suborders: Monocnidea and Dicnidea. The former have spores with single polar filaments, while the spores of the latter have two polar filaments. There are three recognized families in the suborder Monocnidea: Nosematidae, Coccosporidae, and Mrazekiidae. The suborder Dicnidea so far is known to contain only one family, Telomyxidae. Of all these families the Nosematidae is probably the most important from the standpoint of insect pathology. It consists of approximately 10 genera, the best known of which is *Nosema*, the genus containing the notorious agent of the silkworm disease pebrine, namely, *Nosema bombycis* Naegeli.

Biological Aspects

One of the foremost American investigators of Microsporidia is Kudo, who has published numerous papers, including a monograph (1924), on the group. Accordingly, any general discussion of Microsporidia will have to rely heavily on his publications as well as on those of certain European investigators, such as Jirovec and Weiser in Czechoslovakia.

Before considering in detail any particular microsporidian infection, it may be well to deal first with a few general aspects of the morphology and biology of these interesting parasites and their relationships to their insect hosts.

The Spore. If one desires to gain a thorough knowledge of any particular species of Microsporidia, one must have an understanding of both the spore stage and the vegetative stage. The spore and its formation are of first importance, however, in the determination and study of microsporidian infections. It usually has distinguishing characteristics that aid in differentiating one microsporidian species from another. The spore is also of great importance in the epizootiology and biology of microsporidian infections, since it is the resistant stage of the parasite and hence is able to tide the organism over periods of unfavorable environmental conditions and during the period between the change of host individuals.

The average microsporidian spore is from 3 to 6 microns long by 1 to 3 microns broad. The size varies with the species, however, from 1.25 microns long by 1 micron wide in *Nosema pulvis* Pérez to 17 to 23 microns long by 3.5 microns wide in *Mrazekia argoisi* (L. & H.). (Both of these

species occur in Crustacea.) The spores of any one species may vary considerably in size even in the same host individual. The size of the spore is probably determined by the size of the schizont and sporont from which it develops. Outside of this general variance in the size of spores of a single species, some species apparently exhibit two distinct spore sizes. Some workers have thought of these two types of spores as having different functions or that the dimorphism is related to differences in the sex of the contained amoebulae, and several authors have designated the large and small spores as macrospores and microspores, respectively. In the case of most species, the significance or cause of this dimorphism in spores is not clearly understood.

Fig. 185. Spores of *Nosema leptophlebiae* Weiser in the midgut of *Leptophlebia vespertina* Linn. (*Courtesy of J. Weiser.*)

The microsporidian spore also varies considerably in form. Usually it is oval, ovoidal, ovocylindrical, or pyriform in shape. Occasionally it may be spherical, reniform, tubular, bacilliform, spiral, crescent-shaped, or comma-shaped.

The spore consists essentially of a spore membrane or covering surrounding a sporoplasm and a polar filament coiled directly in the spore or encased within a polar capsule. The spore membrane is a highly refractive structure of uniform thickness, its outer surface usually being smooth and structureless. This last character is a useful one in differentiating microsporidian spores from those of Myxosporidia in which characteristic striations frequently occur. Another difference between the two groups is that the membrane of the myxosporidian spore is composed of two valves, whereas that of the microsporidian spores, with a few exceptions, is believed by most observers to consist of a single piece. The Myxosporidia, however, have not been observed in insects. The chemical constitution of the spore membrane is not known with certainty, but in some species it behaves very much like chitin when subjected to mineral acids (Kudo, 1924).

Internally, as has just been indicated, the typical microsporidian spore contains a sporoplasm and a polar filament. The finer structure of these parts and their exact arrangements are thoroughly known for only a few

species, and in certain other species there is so much diversity of opinion on these points that the picture is anything but clear. In many species two vacuoles or clear spaces are discernible, one at the narrow, or anterior, end and the other at the broad, or posterior, end. In some species only the vacuole at one end may be seen, although at times, in old spores, even this vacuole cannot be seen. Usually, however, when the spore membrane

Fig. 186. The appearance of microsporidian spores (*Nosema*) in cross sections of the fat tissue of an infected insect. In this case the host is a hymenopterous insect, *Macrocentrus ancylivorus* Roh. (*Photograph by K. M. Hughes.*)

is thin, the vacuole can be seen in the spores of most species. The sporoplasm or amoebula frequently occupies the central region of the spore, between the two vacuoles. Actually, the sporoplasm has the form of a ring or girdle placed slightly nearer the anterior than the posterior vacuole. Apparently two types of polar filament arrangement exist: the filament may be confined to a polar capsule (usually at the anterior end) or it may be coiled in an area of the spore itself.

The polar filament is an extremely fine, long structure that may be caused to extrude from the spore by a variety of means other than that which takes place naturally in its host. The application of mechanical pressure, acids, iodine, glycerin, and other substances will serve experi-

mentally to bring about this extrusion in some species. Filaments of over 500 microns have been recorded in certain species, but one of the longest in entomogenous species is that of *Nosema apis*, the filament of which varies from 230 to 400 microns in length. In most instances the filament is fine throughout, although in the genus *Mrazekia* the filament is composed of two portions, one of which is a rather thick basal rodlike structure, the "manubrium," and the other a uniformly fine filament. In most other species, the end with which the extruded filament is attached to the spore is thickened and somewhat rounded in shape. The free end of the filament usually tapers to a point. Whether the filament of most species is characteristically hollow or solid has not been conclusively demonstrated. The small opening, or foramen, through which the filament is ejected is usually located at the narrow or tapered end of the spore. In some species this opening is located at the tip of the narrowed end, but in others it is to one side of the tip end. When first extruded, the filament shows numerous windings, crooks, and turns; soon, however, it straightens out. The extruding process may take place very rapidly, or it may occur rather slowly—in either case the process causes the spore itself to undergo a vigorous vibration. The mechanism by which the extrusion is brought about is not known with certainty, but the process may be due to some physical force or pressure built up within the spore.

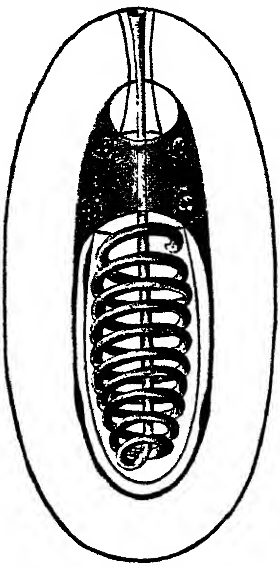


Fig. 187. A diagrammatic representation of the spore of *Nosema bombycis* Naeg., greatly enlarged. (*From Stempell, 1909.*)

The exact function of the polar filament is still a debatable subject, but the structure is thought by some to be an attachment apparatus anchoring the spore to the gut epithelial cell. It has also been supposed that the filament serves to conduct the sporozoite to a distant part of the gut or tissue infected. In this way the advance of the parasite into fresh areas may be ensured. Ohshima (1937) believes that the polar filament, in the case of *Nosema bombycis* at least, is a germination tubule. Immediately after the spore extrudes its filament it discharges a viscous fluid through the tube of the filament. Ohshima believes that the viscous fluid is the amoebula itself. In the silkworm the spore evaginates its filament through the peritrophic membrane into the epithelium. Thus the germ is safely poured out at that point through the tubule of the

filament, protected on its way from the digestive ferments of the alimentary canal. Further substantiation of these observations is needed.

The Vegetative Form. Soon after a microsporidian spore is ingested by a suitable host, the digestive fluids of the latter influence the spore so as to cause it to extrude its filament. The filament becomes detached from the spore, leaving a small opening, or foramen, in the spore membrane. Most observers report that it is through this opening that the sporoplasm creeps out as an amoebula. Some workers have noted that sometimes the sporoplasm emerges from the side of the spore, presumably through a rupture of the spore membrane. Except for Ohshima's observations, mentioned in a preceding paragraph, most investigators believe that the sporoplasm emerges from the foramen into the lumen of the host's gut and by amoeboid movements penetrates the intestinal epithelium.

The next phase of the protozoan's activity is not clearly known for very many species. In some cases it is believed that the sporoplasm creeps about over the intestinal epithelium a while before it penetrates this tissue. In other cases it is thought that the amoebula penetrates through the intestinal epithelium and, for a time, leads an extracellular life in the hemocoele or in the intercellular spaces of the insect's body before commencing its intracellular development. In any case, the greater part of the multiplication and sporulation periods take place within the host cells, the site of which may vary according to the selectivity of the parasite.

Most protozoologists refer to the early intracellular stages as "schizonts," but some call them "meronts." These trophozoites grow at the expense of the host cell. They are more or less rounded bodies, incapable of movement, and they ordinarily possess one nucleus. Frequently the parasite in the cell is surrounded by a narrow clear space. After growing to a certain point, the schizont undergoes a division, usually binary fission, and produces two daughter schizonts. Unequal binary fission, occasionally seen, is sometimes referred to as "budding." As Kudo (1924) has pointed out, under certain conditions and in some species, the division of the nucleus is not directly followed by the complete separation of the cytoplasm. In such cases the nuclei divide further, producing various chain or sausage forms.

The period of schizogony ends in the formation of sporonts, each of which produces a single or a number of sporoblasts and then spores. This is the sporogony part of the life cycle. In most genera the sporont grows, its nucleus dividing into 2, 4, 8, 16, or more daughter nuclei, each of which becomes the nucleus of a sporoblast, and thus each sporont produces a number of sporoblasts. Each sporoblast becomes a spore. In the genus *Nosema*, however, only one spore is produced from each sporont.

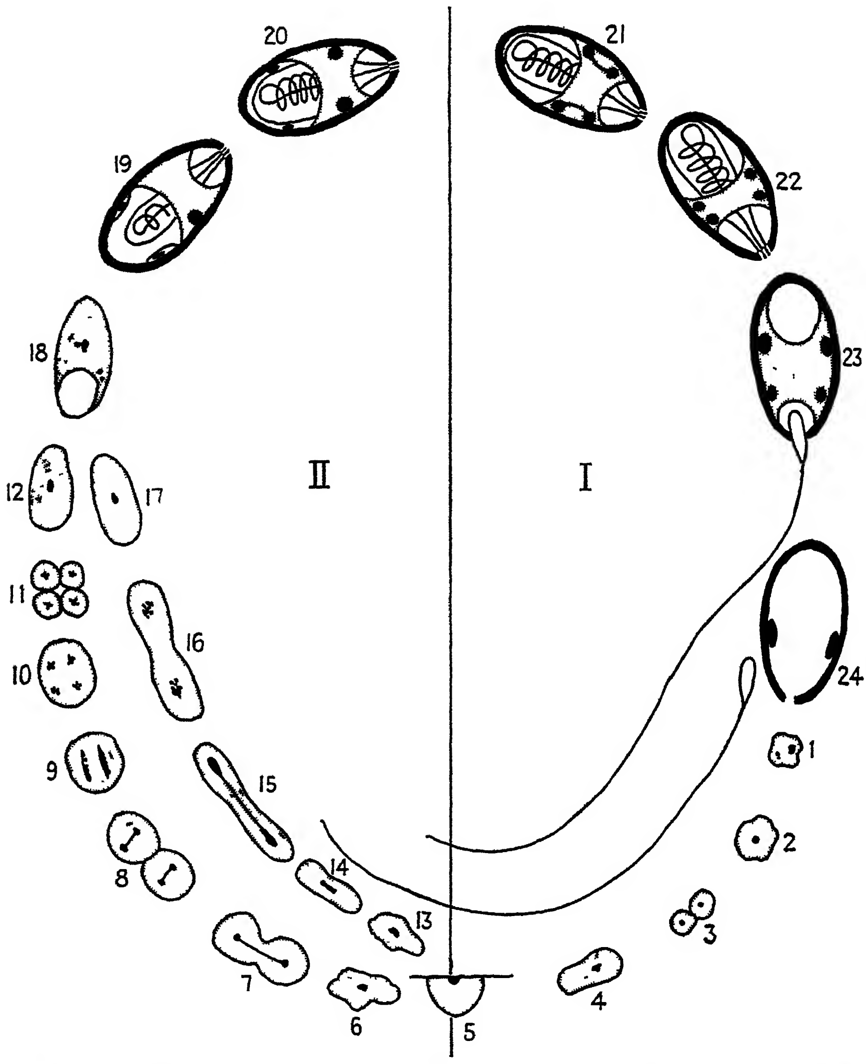


Fig. 188. Diagrammatic representation of the life cycle of *Nosema bombycis* Naeg. in the silkworm. I. Extracellular stages. II. Intracellular stages. 1–4. Planonts. 5–17. Meronts. 18–20. Stages in sporulation. 21–22. Spores in midgut of new host. 23. Extrusion of polar filament. 24. Amoebula leaving spore. For further explanation refer to text. (*Adapted from Stempell, 1909; drawn by K. Snyder.*)

By way of recapitulation, no better example of the life cycle of an entomophilic microsporidian can be provided than that of *Nosema bombycis* Naegeli, the cause of pebrine in the silkworm, *Bombyx mori* (Linn.). The life history of this protozoan has been studied by Stempell (1909), who

gives for it the following life cycle: Soon after the spore has been ingested by a silkworm, the two nuclei of the sporoplasm divide once, producing four nuclei of equal size. The polar filament is then extruded and becomes detached, after which a small binucleated sporoplasm creeps out, leaving behind the other two nuclei which degenerate in the spore. The two nuclei of the freshly escaped amoebula soon fuse into one, and a uninucleated "planont," as Stempell calls it, is formed. These planonts pass between the epithelial cells of the insect's intestine into the hemocoele, where they multiply by binary fission. They are soon distributed to the various tissues of the body, including the ovary, where they become schizonts or, as designated by Stempell, meronts. In their intracellular location these meronts are spherical or oval in shape and divide actively by fission, budding, or multiple division. The individuals resulting from this multiplication are frequently arranged in more or less beadlike parallel rows. Eventually the cytoplasm of the host cell is exhausted and the cell is almost completely filled with schizonts. Each meront then becomes transformed into a spore. In each spore four nuclei are formed originally, two of which, with some of the cytoplasm, form the spore capsule or membrane. Of the remaining two, one is for the polar capsule and the other becomes the nucleus of the sporoplasm. This nucleus later divides, giving the sporoplasm two, and still later (*i.e.*, after being taken up by a new host), four nuclei. The entire life cycle is usually completed in about 4 days.

Microsporidian Species and Host Distribution. A rather hasty perusal of the literature reveals that over 100 species of Microsporidia have been named and described from insect hosts. Most of these are of the genera *Nosema* (about 40 species) and *Thelohania* (about 30 species), with fewer numbers in the genera *Plistophora*, *Perezia*, *Cocconema*, *Gurleya*, *Mrazekia*, *Bacillidium*, *Octosporea*, *Stempellia*, *Glugea*, *Toxoglugea* (= *Toxonema*), *Spiroglugea* (= *Spironema*), *Dubosqia*, *Trichodubosqia*, *Caudospora*, and *Telomyxa*. We find that when the members of the suborder Monocnidea are considered as families, the family Nosematidae includes about 85 entomophilic species; Coccosporidae, about 4 species; and Mrazekiidae, about 9 species. In the suborder Dicnidea apparently only one species has been reported from insects, *Telomyxa glugeiformis* L. & H. from the fat body of the larva of *Ephemera vulgata* Linn. A considerable number of additional microsporidia have been observed in insects but have not been identified or described. It is perhaps possible that if these were known it would bring the number of named entomophilic species to more than 150.

The insect hosts of these microsporidia are included in more than a dozen orders of Hexopoda. In numerous instances the same host species

is known to be the natural host of several species of microsporidia. The following is a list of the approximate number of different species in each of the insect orders[1] concerned that have so far been recorded as hosts of microsporidia. The numbers given refer to the natural hosts only and do not include the species (15 or 20) which have been used as experimental hosts.

Diptera	45	Isoptera	2
Lepidoptera	25	Plecoptera	2
Ephemerida	13	Anoplura	1
Hymenoptera	6	Hemiptera	1
Trichoptera	3	Odonata	1
Orthoptera	2	Siphonaptera	1
Coleoptera	2	Thysanura	1

In addition to the number of species indicated in this list there may be added a considerable number of insect hosts, at least 30, in which microsporidia have been observed but not identified. For the names of some of these insects the reader is referred to Kudo (1924).

With all due respect to those relatively few protozoologists who have worked on the systematics of Microsporidia, it should be pointed out that the group still needs a great deal of thorough revision and validation. This is particularly true at the generic and specific levels. There has been a tendency to describe as new and separate species similar-appearing microsporidia merely because they occurred in different host species. Some specialists have expressed the view that in nature the specificity of any particular parasite for a host varies somewhat but that usually a species of microsporidia will infect only those insects within a single genus or possibly a few in closely allied genera. *Thelohania legeri* Hesse, for example, has been found in five species of *Anopheles* mosquitoes, and *Thelohania opacita* Kudo has been observed in three species of *Culex* mosquitoes. According to Kudo, the host species of these two genera of mosquitoes have been seen in many instances living side by side in the same waters, yet not a single case of mixed infection has been discovered. It is also probably true that some species of microsporidia parasitize only a single specific host species. On the other hand, under experimental or other specialized conditions, insects quite different from the natural host may become infected with a particular microsporidian. Steinhaus and Hughes (1949), for example, found at least 10 different insect species in three different orders (Lepidoptera, Hymenoptera, and Neuroptera) to be susceptible to a microsporidian (*Nosema destructor* S. & H.) originally

[1] Since some European entomologists and zoologists use a different system from that of Americans for classifying insects into orders, these approximate figures are subject to reinterpretation depending on the system of classification used (*e.g.*, see Weiser, 1947).

observed in the potato tuberworm, *Gnorimoschema operculella* (Zeller). Whether or not such wide susceptibility occurs in nature is not known. It is clear, however, that there is considerable danger in the practice of considering as a new species each microsporidian found in a new host.

Another disquieting feature of microsporidian systematics is the indefiniteness of the distinguishing characters that are used to separate not only species but genera as well. One cannot help but feel the insecurity inherent in separating genera (*e.g.*, *Glugea* and *Perezia*) not on the basis of their own characteristics but largely upon pathological changes occurring within the tissues of the host. Similarly, upon consulting the original literature concerned with the description of certain of the species of *Perezia*, for example, one is inclined to question the validity and identity of some members of the genus. Furthermore, since several species of *Nosema* (including on one occasion at least the type species, *N. bombycis*) have been described as *sometimes* producing two spores from one sporont, it is difficult to understand at just what point these should be separated from species of *Perezia* described as *not always* forming two spores from one sporont. Or perhaps the genus *Nosema*, as it is now known, is too large and inclusive. The ease with which in the past some authors have identified species with which they worked as *Nosema* suggests that this genus is not now properly constituted. Perhaps when someone undertakes to restudy all the known species of this genus, the situation will become clarified. In the meantime, the student should be aware of the taxonomic difficulties with which the microsporidia are afflicted. Fortunately techniques used by Weiser and certain other contemporary specialists of Nosematidae aid in clarifying some of these difficulties. For example, with some species of true *Perezia*, at least, the two spores are found consistently to lie side by side in slide preparations from diseased insects. Extremely thin smear preparations of these *Perezia* show the spores always in pairs; similar smears of *Nosema* show the spores to occur singly. The situation is not always so clearly defined, however, and some protozoologists distinguish the various genera on the basis of the predominant type of spore formation. If most of the sporonts yield one spore, the species concerned is considered to belong to the genus *Nosema*; if the greater percentage of sporonts produce two spores, it is a *Perezia* or *Glugea*; four spores from each sporont indicates the genus *Gurleya;* eight spores indicate *Thelohania;* etc. In any case considerable actual experience is a prerequisite to diagnosing accurately both species and genera of Microsporidia. The picture will undoubtedly become more clear as the new systematic concepts and descriptions continue to supersede the old.

The geographical distribution of most species of microsporidia is only partly known. Some species are widely distributed and are known to

occur in nearly every country where their natural hosts live. *Nosema bombycis* Naegeli in the silkworm and *Nosema apis* Zander in the honeybee are examples of this wide distribution. On the other hand, some species occur only in certain general localities or are limited to definite small areas. Thus Paillot (1918*a*) in France found *Perezia mesnili* Paillot to be present in *Pieris brassicae* (Linn.) in only certain areas. What the factors are that determine the geographical distribution of entomophilic microsporidia are only slightly known.

Although the seasonal distribution, as it concerns most species of microsporidia, is still largely uninvestigated, it is known that in some instances the incidence of infection seems to be high in summer and low in winter.

Diseases Caused by Microsporidia

General Considerations

Before considering in detail some of the various microsporidian infections, it may be well here to set forth briefly a few of the more general aspects of the microsporidioses in insects.

Methods of Infection. The most common natural route by which susceptible insects are infected by microsporidia is through the mouth. It has already been pointed out that the spore, soon after being ingested by a suitable host, extrudes its polar filament, which becomes detached, and soon thereafter the infecting sporoplasm creeps out. This amoebula then infects its host via the gut. The use of the oral route as a mode of infection has been confirmed experimentally.

Once established in its host, the microsporidian may reinfect tissues of the same host by repetition of development. This autoinfection has been recorded for several species.

Just how many species of microsporidia are capable of being transmitted transovarially from one generation of the host to the next has not been determined. That such means of transmission and infection are possible was shown as early as 1870 by Pasteur during his work on *Nosema bombycis* in silkworms. Subsequent investigators have noticed this method of transmission in the case of other species. Several reports tell of the infection of ovary and eggs by the sporozoan, and there is little doubt that it is a rather frequent occurrence. Such a mode of infection is especially likely to succeed if the infection is slight or moderate, at least mild enough so that it does not affect the development of the host embryo.

General Symptoms and Gross Pathologies. The external appearance of a microsporidian-infected insect may vary according to the degree and extent of the infection. Changes in color, size, form, and activity may be evident separately or together.

One of the most common color alterations is the change from transparency of the body to opacity, usually of a dull milky-white appearance. This opacity is due to the accumulation of large numbers of spores in the tissues underlying the insect's integument. In addition, dark mottled areas or spots of dark-brown coloration may appear. Sometimes a grayish or yellowish color is assumed by infected individuals; even a red coloration due to microsporidia has been recorded.

Changes in body form frequently accompany the abnormal changes in color. The host may remain small or dwarfed as the result of infection, or it may become distended or swollen. Actual deformities have also been noted. Such changes naturally affect the activity of the host and its reaction to external stimuli. The activity of the insect becomes impaired either because the muscles themselves are infected or because the muscles are pushed aside by pressure against them caused by the distention of infected tissues, such as the fat body, or by the presence of large numbers of spores. Infected insects may complete their metamorphosis, but instances are known in which infected larvae are unable to pass into the pupal and adult stages.

The internal gross pathology also depends upon the degree and extent of the infection. In numerous cases only a specific tissue is invaded by the parasite, and in other cases nearly all the tissues of the body are affected. It appears that in arthropods the fat body and other adipose tissue are one of the favorite seats of infection by microsporidia. This tissue is relatively large and furnishes an abundant supply of nutriment for the protozoan. Other tissues in the same host may be affected; but because of the prominence of the fat body and any changes brought about therein, it is the most frequently noticed diseased tissue. As a result of the parasite invasion, the fat tissue itself may become considerably diminished in size. In fact, in some cases the fat body may be completely replaced by the parasite; as a result, the animal usually dies. Occasionally the diminution of the fat tissue may be accompanied by an increase in the quantity of hemolymph.

Histopathology. Perhaps the most noteworthy change frequently brought about in the tissues of insects parasitized by microsporidia is the enormous englargement of cells. The nuclei of the infected cells may become hypertrophied, and they may increase in number, during which process they may exhibit various types of division. The cytoplasm of the cells also becomes enlarged principally because of the distention caused by the large number of parasites. In some instances the cytoplasm may entirely disappear, and as the cell enlarges the nucleus also undergoes hypertrophy. Chromatolysis and karyolysis may occur simultaneously, the chromatin material gathering in masses at the periphery of the nucleus. The cell

membrane usually remains intact until the death of the tissue or the insect.

In some cases the leucocytes of the host act against the parasites, but the phenomenon of phagocytosis has been given attention in only a few instances of microsporidian infection.

Whether or not microsporidia produce a toxin that affects the host has not been determined, but such a possibility remains. Similar uncertainty prevails concerning the production of an immunity by the host against the invading parasite. There are indications that certain races of silkworms, for example, have an inherent resistance or lack of susceptibility to infection by *Nosema bombycis*. The resistance of certain breeds of bees to microsporidian infection has been debated but still is undecided.

Pebrine
(Microsporidiosis of the Silkworm)

Pebrine (French *pébrine*) is the name most frequently used to designate the microsporidian disease of the silkworm, *Bombyx mori* (Linn.), caused by *Nosema bombycis* Naegeli. The disease has been known by numerous other names. Some of these were derived directly from Latin, but many were derived from the native language of the originator or arose as colloquialisms. Thus we have names in the French, Italian, Spanish, German, Japanese, Hindi, and other languages that have been used to refer to the disease. They include such designations as *maladie des petits*, *étisie*, *maladie corpusculeuse*, *idropisia della farfalla*, *malattia dei corpuscoli*, *mal delle petecchie*, *atrofia contagiosa*, *necrosi*, *segno nero*, *dystrophia mycetica*, *acetotrophie*, *Körperchenkrankheit*, *Fleckenkrankheit*, *kokushibio*, *biriushibio*, and *kata*. Most names such as these are of historical interest only, and it is not at all unlikely that some of them really describe disease conditions other than pebrine; *e.g.*, muscardine. In this book we shall use the name "pebrine" keeping in mind Plato's words that "They do certainly give very strange and new-fangled names to diseases."

The name *pébrine* was given to the disease in 1860 by de Quatrefages because a characteristic symptom of the infection is the appearance of dark pepperlike spots on the integument of the diseased silkworm. As one author wrote, "The spots on the diseased worms are, in fact, rather like pepper grains." The name is derived from the Provençal word *pebrino*, which is from the patois form *pébré*, meaning pepper. *Pébré* is from the Latin *piper*.

Early History. Pebrine has probably existed since very remote times, but one of the first recorded outbreaks of the disease that might be recognized as such appears to have occurred in 1845 in the province of Vaucluse, France, at Cavaillon in the valley of Durance near Avignon.

The disease soon spread to, or was recognized in, the nearby countries of Italy, Spain, Turkey, and Syria, in the provinces of Walachia and Moldavia in Rumania, and in Caucasia and China. By 1864 all the silkworm-rearing countries of Europe and some of those in Asia were unable to rear uninfected insects with any degree of certainty. Japan alone appeared to remain free of the blight, although the parasite was later to be detected in this country as well. It is worthy of note that Japan gained her renowned supremacy in sericulture by virtue of the outbreaks of disease in the silkworms of Europe. The entire situation was critical, especially as it affected the production of disease-free eggs. As soon as the silkworm cultivators in a particular area found their stocks infected, they searched for new sources of eggs (*graine* or "seed") in disease-free areas. In 1865, for example, it has been estimated that 3 million egg cards (each card carried 1 ounce of "seed") were imported by France and Italy from Japan. The disease-free areas became increasingly limited in size and in number. The situation went from bad to worse, until finally sericulturists demanded that remedial measures be taken to cope with the problem. In 1853, while rearing was being maintained by imported eggs, silk production in France amounted to 26 million kilograms of cocoons valued at 117 million francs. In 1865, when the pestilence was extremely severe, the production dropped to 4 million kilograms. In the 13 years following 1853, the estimated financial loss amounted to 1 billion francs. During a similar 10-year period in Italy the loss was 2 billion francs. Special commissions were established both in Italy (1856) and in France (1858), but not a great deal was accomplished toward eradicating the disease. As will be explained when we discuss the causative agent of pebrine—and this phase of the subject has a history of its own—no one at this time had a clear idea as to the true cause of the disease. Lacking this information it is understandable that very little progress was made in attempting to solve the problem.

Then in 1865 the French Senate received a petition signed by more than 3,500 sericulturists from the silk-producing areas of France. The petition requested that the government (1) reduce the taxes (always a logical request!); (2) place at the disposition of the sericulturists a superior strain of eggs; and (3) provide for a study of the silk-raising industry from the standpoint of both pathology[1] and hygiene. Their cause was championed by J. B. Dumas, who presented to the Senate, of which he was a member, a report on the dire situation facing the silk industry of France. While preparing his report for the Senate, Dumas thought of

[1] ". . . tant au point de vue de la pathologie qu'à celui de l'hygiène." It is of interest that the term "pathology" was being used in connection with the diseases of insects at this early date (1865).

his friend and former pupil Louis Pasteur and importuned him to undertake the research necessary to discover means of checking the ravages of the disease.

Pasteur had just discovered the cause of the "diseases" of wine and had been instrumental in dealing the deathblow to the concept of spontaneous generation. He hesitated to leave his laboratory and work, but the gravity of the situation and his friendship for Dumas impelled him to undertake the project. As translated by his biographer, Vallery-Radot, Pasteur wrote to Dumas:

> Your proposition throws me into a great perplexity; it is indeed most flattering and the object is a high one, but it troubles and embarrasses me! Remember, if you please, that I have never even touched a silkworm. If I had some of your knowledge on the subject I should not hesitate; it may even come within the range of my present studies. However, the recollection of your many kindnesses to me would leave me bitter regrets if I were to decline your pressing invitation. Do as you like with me.

He was commissioned to undertake the study of silkworm diseases by the Minister of Agriculture, and the Empress Eugénie extended him her good wishes and further urged him to take up the problem—to which Pasteur then promised to devote 5 years of intensive research. Except for his loyalty to God and his love for his family, patriotic Pasteur placed the welfare of his beloved France above all else. Thus began the real scientific development of insect pathology, and no more illustrious scientist could have been destined to begin it!

Pasteur began his work on June 16, 1865, by going to Alais in the department of Gard, which was the most important mulberry-raising part of France and the area in which the disease was the most serious. By thoroughly questioning the Alaisians and by constant observations, Pasteur familiarized himself with the details of sericulture. Until he began these studies he hardly knew what a silkworm looked like (" . . . je n'avais pas encore eu l'occasion de voir le précieux insecte"). The natives of Alais told him of innumerable remedies for the control of pebrine and furnished him with many theories as to its nature and cause, including the pertinent observation that it was something like the plague or cholera. He submitted a report of these observations to the Academy of Sciences in September, 1865. His work was momentarily interrupted by the death of his father and later by the death of his youngest daughter. Added to this was the discouragement of skeptics and the pessimism of most of the silkworm cultivators of Alais, who were disappointed that the government would choose a "mere chemist" for this important task rather than a recognized zoologist or sericulturist. In 1866 Pasteur returned to Alais with two

assistants, Gernez and Maillot, and set up a laboratory in a lonely house at the foot of the Mount of the Hermitage. Here Pasteur began his intense labors, burdened with the death of still another of his daughters, and here he made most of his discoveries concerning the nature of the disease.

Some years prior to this time, in 1859 and 1860, de Quatrefages and his commission published the results of their investigations into the nature

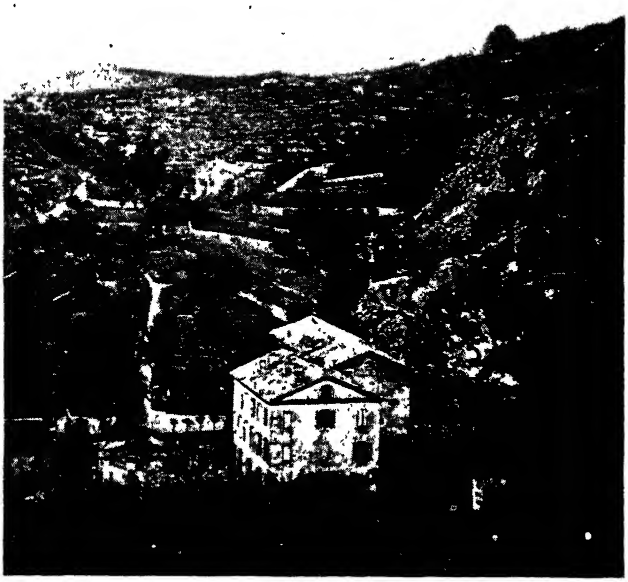


Fig. 189. The house (in the foreground) near Alais, France, where Pasteur did his early work on pebrine and flacherie of the silkworm. (*Reproduced from cut in Pasteur's (1870) book on the diseases of the silkworm.*)

of the diseases of the silkworm. These reports were thoroughly studied by Pasteur. In the first report, one passage in particular attracted his attention. This was a paragraph having to do with the peculiar microscopic corpuscles that a number of earlier workers had seen in the bodies of diseased silkworms. To determine the significance of these corpuscles was Pasteur's first objective. Before long this French investigator established (1) that the corpuscles are special characteristics of the disease and that they invariably manifest themselves, if not in the larvae and pupae, then in the mature moths; (2) that the corpuscles are parasites and are not only a sign of the disease but its cause; and (3) that the disease may be transmitted through the egg, by contact with diseased silkworms, and through the ingestion of contaminated food. Pasteur concluded

that the disease was actually inherent in many successive generations of the silkworm and that the epizootic condition was only an exaggeration of a normal state and brought about by the particular methods of cultivation and production of eggs being used.

Pasteur's studies led him to the investigation of another of the great scourges of sericulture, flacherie. Finally, in 1870 he published his famous memoir, "Etudes sur la maladie des vers à soie," which concerned itself with both pebrine and flacherie. This report constitutes a gem among all scientific publications not only because of its importance to the study of disease generally but also because it reveals the excellence with which this great scientist accomplished his work. It also reveals much of the human side of Pasteur. At times confident and somewhat boastful, at other times he was uncertain, humble, and modest. A pleasant mixture of these traits may be detected in the first sentences of the preface of his memoir. He commenced by writing "I should begin this work by excusing myself for having undertaken it." This he does on the basis that he was ill prepared to pursue such research, the subject of which was so unfamiliar to him. One can imagine that it was with a deep feeling of understandable pride that he wrote these words, knowing that he was presenting to the world the fruits of a great success—even though he began inspirationally handicapped and uninformed.

Pasteur finished his work on this problem by furnishing a method by which the diseased eggs could be detected and eliminated, thus ensuring the silkworm cultivators a healthy supply of insects. By following his methods, or slight modifications of it, sericulturists have done much to bring pebrine under control, and it has largely ceased to be the extreme menace it once was. At the present time the silk-producing countries of the world only occasionally suffer outbreaks of the disease, and these are usually of limited magnitude and are brought rapidly under control.

We cannot refrain here from pointing out that it was during his work on the diseases of the silkworm that Pasteur gained his insight into the basic phenomena of infection. It was this experience and self-training which later was to give him background and incentive to delve into the mysteries of animal and human diseases. To the writer, it seems that when Pasteur's great medical discoveries are extolled, it is too often forgotten that, had it not been for his work on the diseases of the silkworm, this French scientist might never have made monumental discoveries on anthrax, rabies, septicemia, and other infectious diseases. To be sure, some other pioneer eventually would have come along to work out these same problems (and chemistry undoubtedly suffered because of Pasteur's diversion), but mankind must extend an acclamation of appreciation to the lowly silkworm for having suffered a disease that commanded the

attention of such a scientist as Pasteur. Had the medical men of that day condescended to be concerned about the affliction of a mere insect, they might not have had to suffer the lasting "embarrassment" of having a nonmedical scientist point the way for them to the modern concept of infectious disease. Medical science also owes a debt not only to Pasteur but to the silkworm and its diseases!

The Causative Agent. As was the case with many other diseases of insects, pebrine was at one time ascribed to a variety of causes. Furthermore the true nature of the disease was frequently confused by the presence of other diseases in the same nursery or by the presence of more than one infecting agent in the same silkworm. Thus the "hematozoïds" described in 1849 by Guérin-Méneville in larvae obviously infected with the fungus of muscardine probably represented the spores of *Nosema bombycis*, indicating a double infection. De Filippi, in 1852, also observed these "hematozoïds," but, like Guérin-Méneville, he did not recognize their true significance. This was accomplished in 1856 by Cornalia, who noted the relation between the presence of the "hematozoïds" and the occurrence of the disease (known to him as "hydropsy") in moths. In the meantime, the disease was being ascribed to such causes as degeneration of mulberry leaves, to mites living on the leaves, to alterations in the blood, to the breakdown of the hemocytes, to atmospheric conditions, and to a variety of miasmic influences.

In 1856 and 1858 Lebert and Frey made important contributions in that they insisted on the pathological significance of the pebrine corpuscles. They considered them to be a vegetable parasite, and in 1858 Lebert gave them the name *Panhistophyton ovatum*. In the year just prior to this, however, Naegeli (1857) briefly described the corpuscles as representing some form of yeastlike fungus and gave to them the present name *Nosema bombycis* (the word *Nosema* coming from the Greek word meaning "disease"). He associated it with a diseased condition in the silkworm as it occurred in France and Italy.

In spite of these reports, the idea that pebrine was a contagious microbial disease was slow in being accepted. Chavannes, in 1862, believed that the disease was due to alterations in the blood, which contained uric and hippuric acids, and that these acids cause the hemocytes to degenerate in such a way that the nucleoli of the blood cells are set free, becoming what observers regarded as the pebrine corpuscles. Brouzet (1863), however, was of the opinion that pebrine was comparable to typhus, in that it appeared in an epidemic, infectious, and contagious form, and that it arose spontaneously "by the alteration of the exhalations" that arise from animals packed into a small space. Although an adherent to the theory of spontaneous generation, Brouzet was striking very close to the

truth, especially when he compared the animalicules seen by Rayer and Davaine in the blood of sheep dead of anthrax with the corpuscles found in the blood of diseased caterpillars in that they were each responsible for the diseased condition concerned. Also to Brouzet's credit is his pronouncement that pebrine would be successfully combated when a means of destroying the corpuscles was found.

Thus we see that the scene was set for Pasteur's thorough and penetrating experiments, which clarified and established once and for all the etiological role of the corpuscles in pebrine. At first Pasteur did not believe that the corpuscles represented separate living entities distinct from morphological elements of the insect's body. He first believed them to be elements incapable of reproduction. He soon changed his opinion, however, and by 1870 he considered them to represent a separate organism which he, in agreement with Leydig, thought should be placed in the group then known as "psorospermie"[1] or in a closely related group. Although he recognized their relation to other psorosperms of that day, Pasteur was mistaken concerning the developmental cycle of the parasite and did not recognize the corpuscles as spores. This fact was, however, recognized by Béchamp. It remained for Balbiani, in 1882, to place the organism formally in the class Sporozoa and to erect a new group, Microsporidia, to include the pebrine organism and related forms. Then, in 1909, Stempell published his well-known paper describing in detail the various stages in the life cycle of *Nosema bombycis*. Today approximately 40 species of the genus *Nosema* have been reported from insects.

In an earlier section (page 586) we described the life cycle of *Nosema bombycis,* using it as an example to illustrate the cycle of a typical microsporidian. Accordingly, it is unnecessary to repeat these details here. Suffice it to say that, under favorable conditions, the entire life cycle, from spore to spore, requires approximately 4 days and in its details is very much the same as is that of most Nosematidae. After the spore is ingested the young amoebula escapes, penetrates the gut epithelium, and enters the hemocoele. From here it migrates to any of the various parts of the body, entering the cytoplasm of a cell. The size of the schizont is about 1.5 to 2.5 microns in diameter. After active multiplication, it eventually is transformed into a single spore. The parasite has never been cultivated in vitro.

The spore—*i.e.*, the "corpuscle" of Pasteur's day—is usually oval or pyriform in shape. In size it generally ranges from 3 to 4 microns in

[1] The word "psorosperms," now rarely used, is a term originally coined by Johannes Müller to denote the spores of Myxosporidia. It was soon extended to other parasitic microorganisms that form spores, such as the gregarines and coccidia, and hence the words "Sporozoa" and "psorosperms" became almost synonymous terms.

length by 1.5 to 2 microns in width. The polar filament, which may be extruded by the action of perhydrol or mechanical pressure, varies from 34 to 72 microns, or even up to almost 100 microns in length. A vacuole can usually be seen at each end of the spore. The spore membrane is probably not thicker than 0.5 micron. The sporoplasm is a girdle-shaped band of protoplasm apparently possessing two nuclei, which later become four. The polar capsule may be made clear by applying nitric acid.

Symptoms of Pebrine. It has already been pointed out that one of the characteristic symptoms of pebrine is the appearance of dark-brown to black spots on the surface of the silkworm's integument. The spots have irregular but distinct contours and occur principally on the posterior ventral side of the insect. These spots, however, usually do not appear until the insect is in an advanced stage of the disease and usually during the fourth or fifth larval instar, or they may appear on infected pupae and adults. Usually, however, other symptoms appear before this.

Fig. 190. The middle segments of a silkworm caterpillar infected with *Nosema bombycis* Naeg., and showing the dark spots characteristic of pebrine. (*From Stempell, 1909.*)

In any particular group of simultaneously hatched larvae the infected insects show irregular growth, particularly as evidenced by size. The irregularity usually shows up after the first or second molt. Some of the infected larvae grow quite normally; others are stunted and diminished in size. Lightly infected larvae do not show many symptoms, but several abnormalities in addition to the dark spots mentioned above may be exhibited by heavily infected individuals. The latter move about sluggishly, lose their appetites, grow very slowly, and frequently die before pupating. The silk from the cocoons of infected larvae usually is much inferior in strength and uniformity of thickness to that of healthy insects.

In addition to the silkworm, other insects have been found experimentally susceptible to *Nosema bombycis*. These include *Arctia caja* Linn., *Margaronia pyloalis* Wlk., *Chilo simplex* (Butl.), and *Hyphantria cunea* (Dru.). In these insects the symptoms are for the most part similar to those of pebrine in *Bombyx mori* (Linn.).

Pathology. *Nosema bombycis* has been reported as occurring in nearly all tissues of all stages of the silkworm. Accordingly the pathological changes are extensive and varied, depending upon the particular

tissues involved. Some studies have been made of the gross pathology, but considerably less work has been done on the histopathology of the disease.

The fat tissue of the silkworm appears to be particularly involved as concerns the pathology of the disease. Furthermore the cells of the silk glands and of the Malpighian tubes are frequently as much affected as are those of the fat body. These three tissues appear to be more susceptible to parasitization by *Nosema bombycis* than do the epithelial cells of the gut and the blood and pericardial cells. Such tissues as those comprising the muscular and the nervous systems are also readily attacked. According to Stempell (1909) the muscular tissue of the infected silkworm becomes liquefied, and microscopic observation reveals it to consist of a homogeneous pasty mass, in which are suspended the remaining muscle fibers and nuclei. The sarcolemma usually remains undisturbed.

In larvae that are rather heavily infected the organs take on a milky-white appearance as a result of the presence of a large number of spores. This condition is particularly noticeable in the silk glands, where the epithelial cells become distended and form irregular tumorlike pustules. The more or less opaque white color of the infected cells contrasts markedly with the translucent normal cells.

It is a curious fact that even though it is heavily parasitized, the host cell may retain its vitality for a long period of time. Some authors believe that this indicates that the parasite does not produce a toxin deleterious to the cell. Furthermore the actual cellular lesions are not very marked, although the undifferentiated cytoplasm of the cell gradually disappears as the parasite increases in number. Frequently only the mitochondria of the cell can be seen between the parasites as they fill the cytoplasmic space. The nucleus is not invaded and usually retains its normal appearance for a considerable length of time after the cell has been attacked. The parasitized cells are generally larger than are the normal cells, particularly in certain tissues such as the hypodermis.

Some authors write that the characteristic black spots visible on the exterior surface of an infected insect are produced by the coagulation of blood after hemorrhage takes place through wounds caused by the microsporidian in the integument. Others believe that, when a hypodermal cell is attacked by the parasite, the chitinous cuticula over the cell becomes brownish in color, and brittle, disintegrating into several fragments. A space is thus formed into which the cell filled with spores drops. The remaining hypodermal cells regenerate and form a continuous layer back of it. When a new layer of chitinous material is secreted by the epithelial cells, the spores enclosed in the space become yellowish in color because of the lack of oxygen. As the result, a spot is produced which appears dark

to the naked eye. In any case, it is known that the parasite is capable of multiplying entirely within the layers of the integument.

Routes of Infection. The most frequent source of infection is that which occurs when the feces from infected worms contaminate the food and are thus ingested by susceptible larvae. The spores contained in the fecal matter easily become spread over the mulberry leaves on which the silkworms feed and hence are unavoidably taken in by the insect with its food.

The second common method and route by which the parasite is transmitted is through the egg. Transovarial transmission has been known since Pasteur's time and in the past has constituted one of the principal means of spreading the disease among sericulture nurseries. The production and sale of contaminated eggs had to be prevented before effective means of control could be instituted.

That the external contamination of the eggs by the parasites is responsible for some of the transmission from one generation to the next is indicated by the observations of Masera (1938*a*). This worker reared healthy larvae from "diseased" eggs after disinfecting the exterior of the eggs. How much of the so-called "hereditary" transmission of the parasite is due to this type of contamination is an interesting point on which to speculate.

Preventive Measures and Control. As in the control of other insects being reared in insectaries or in small colonies, certain general measures can be exercised that facilitate the control of pebrine. These include such things as the maintenance of strict sanitation, the frequent and careful inspection of stocks for signs of disease, the destruction of diseased material, and the proper regulation of temperature and humidity. The proper control of temperature and humidity are especially important for supressing not only pebrine but other diseases as well. The growth of the parasite in the insect appears to be accelerated by extremely high temperature and humidity. At the same time these conditions seem to weaken the general resistance of the host to the parasite.

The method of control developed by Pasteur was the single factor most important in saving the silk industry of France and that of other countries as well. In brief, the method is simply this: The moths are separated in pairs; after the female finishes oviposition it is examined microscopically for the presence of microsporidian spores. The eggs from the moths showing infection are destroyed, while those from healthy moths are retained and used for rearing purposes. Samples of the eggs may also be examined for the parasite. Machines have been devised that crush 25 or 30 moths at a time; the fluid extracted from the bodies of the crushed insects is then examined microscopically for the spores of *Nosema bombycis*. Some-

times the fluids from the moths are first centrifuged and the precipitate examined for the presence of spores; in this way relatively small numbers of the parasite may be detected. Various methods have been employed for examining eggs directly. Most of these methods consist essentially of triturating the eggs in an aqueous solution, clearing with potassium hydroxide, neutralizing with hydrochloric acid, and examining the centrifuged sediment for the presence of spores. Separation of "clean" from "diseased" eggs has been advocated on the basis of the susceptibility of the latter to mercuric vapors (Masera, 1938*b*, 1940). Other methods for detecting or eliminating diseased eggs have been suggested, but most of these are either not very effective or too difficult to put into operation.

It would be of considerable interest to know if the method developed by Allen and Brunson (1947) to rid their potato-tuberworm (*Gnorimoschema operculella* (Zell.)) colony of a *Nosema* infection would be applicable in the case of *Nosema bombycis*. These workers placed the eggs from infected female moths in a watertight metal envelope and immersed this in a hot-water bath at 47°C. for 20 minutes. By this procedure, which destroyed the protozoan but did not harm the egg, they were able to reduce the infection in their host stocks by 75 to 90 per cent. The practicability of the method has been confirmed by Finney, Flanders, and Smith (1947).

Some sericulturists believe that the different breeds or strains of silkworms vary in their natural susceptibility to pebrine and that hence considerable protection against a devastating outbreak of the disease may be obtained by rearing resistant insects. It appears, for example, that the Japanese strains of *Bombyx mori* have a greater resistance against the effects of *Nosema bombycis* than do most other strains. The Japanese silkworms will spin cocoons even though they are infected. Other strains may sometimes be able to spin cocoons, but the silk threads of such cocoons are usually very irregular in thickness and of so little strength that they break easily in reeling.

Nosema Disease of the Honeybee (Microsporidiosis, or Nosemosis, of the Honeybee)

Nosema disease of the honeybee, *Apis mellifera* Linn., is caused by *Nosema apis* Zander, a microsporidian parasite of the intestinal epithelium of adult bees. From the standpoint of the apiary as a whole, the disease is not a serious one, especially when compared with the foulbroods. Individual bees, and sometimes colonies, die from its effects, but rarely, if ever, is an entire apiary destroyed. Some investigators are inclined to believe that *Nosema apis* is an incidental rather than a primary cause of the affliction and that it thrives in bees that are in poor condition from some other cause. In any case the presence of the protozoan in a colony

bodes no good, and some authorities (*e.g.*, Farrar, 1947) maintain that throughout the beekeeping industry the losses, measured by reduced production of honey, attributable to nosema disease may equal or exceed those caused by the better known brood diseases.

Early History and Name of Disease. Bees were probably being parasitized by *Nosema apis* even before man began to keep honeybees. In this sense the disease is not a new or recent one, although its cause and characteristics have been elucidated only during the past century. Before this time the disease was probably confused with some of the other abnormal conditions found in honeybees.

In 1857 Dönhoff reported that with the aid of a microscope he had observed small oval bodies in the digestive tracts of bees that he thought had died of exposure. He sent some of the bees to Leuckart, who, after examining them, thought that the oval bodies were the spores of a fungus. Accordingly, Dönhoff referred to the disease as "Pilzsucht," *i.e.*, "fungous disease." These observations were reported by Dönhoff (1857) in the form of a note to which was appended a letter from Leuckart.

Fifty years passed before significant progress was made on the disease. Then in 1909 Zander reported his finding of small oval bodies in the stomach walls of affected bees. To these parasites he gave the name *Nosema apis,* and in 1911 he called the disease "Nosema-seuche." It is from the English translation of the latter name that we get the present-day designation "Nosema-disease" or "nosema disease." The name is now widely used, but in using it one should make clear that one is speaking of nosema disease *of the honeybee* since other insects are infected with other species of microsporidia of the genus *Nosema.* Linguistic variations of the name nosema disease are also used in Switzerland ("Nosemakrankheit") and Denmark ("Nosemasygdommen"). Zander (1909) originally referred to the disease as a dysentery, but such a connotation is not correct according to our present concept of the dysentery diseases. One objection to the name "nosema disease" may be that it is the Greek-English equivalent of saying "disease disease" which, by itself, is meaningless. The idea here, of course, is that the disease is one caused by a species of the genus *Nosema.* Accordingly, some authors apparently feel that the term used should be in the form "*Nosema*-disease" (*i.e.*, that the italicized form of the generic name should be used).

Following the work of Zander, numerous accounts of the disease appeared. One of the most clarifying of these was that of White (1919), to whom we are indebted for much of our basic information on the disease as it occurs in the United States.

Symptoms and Pathology. A colony of bees that shows weakness, especially in the spring of the year, should be suspected of suffering from

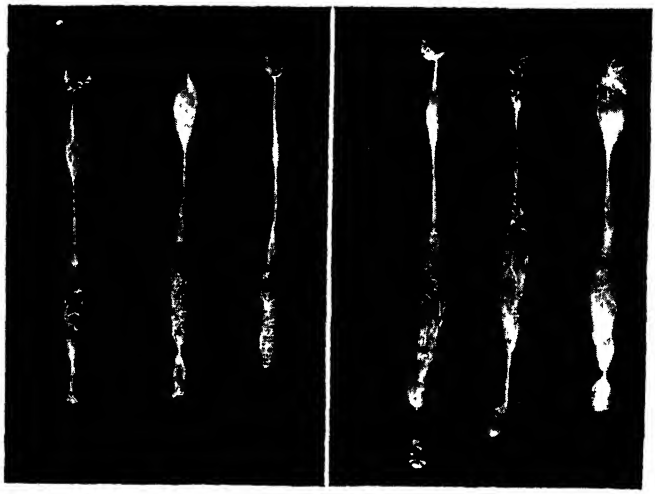
nosema disease. In such colonies parasitized bees can usually be found without much difficulty, and one might recall with Marcus Aurelius that "what is not good for the swarm is not good for the bee." Especially is the colony weak if a large number of bees are infected and if the infection has persisted for a sufficient length of time. Otherwise the behavior of the *Nosema*-infected colony is usually similar to that of a healthy one. The brood is generally normal in appearance; only the adult insect is infected. In markedly weakened colonies the brood sometimes increases to an amount that cannot be properly cared for by the adult bees present.

Nosema disease in the honeybee exhibits very few distinguishing symptoms. According to White (1919), an infected bee manifests no outward symptoms of the disease when seen among the other bees of the colony. Other authors, however, have described infected bees as crawling feebly about on the ground, unwilling to fly or to sting. When able to fly, they do so for only a few yards, then alight. When attempting to begin a flight from the alighting board, infected bees may fall from the board to the earth and die. The abdomen of the bee is often distended and sometimes peculiarly softened. The wings may become dislocated and askew. The legs may be dragged along in crawling, as if they are paralyzed. Some of these symptoms described in certain earlier reports may not be those of nosema disease as such; the condition may have been complicated by the presence of other disorders. In general, it may be said that lightly infected bees show few if any symptoms, whereas heavily infected bees exhibit impairment of mobility and general weakening. The principal outward effects are those affecting the colony as a whole. Valuable notes on the diagnosis of nosema disease of the honeybee (as well as most of the other diseases of this insect) have been presented by Dade (1948).

The gross pathology of the disease is very distinctive. If the head of a worker bee, the caste most often affected, is removed, one may then withdraw the alimentary canal by grasping the very tip of the abdomen and pulling. If *Nosema apis* is present, the ventriculus or midgut is usually white and opaque in appearance, and it may be somewhat swollen. The circular constrictions present in the intestines of healthy bees become more or less obliterated. The stomach tissues of an infected bee are fragile and easily torn. When the tissues are crushed or triturated in a drop of water or saline, a milky-white debris results.

Usually the only tissue involved in the pathology of the disease is the gut epithelium. Occasionally, but rarely, the epithelial cells of the Malpighian tubes are parasitized. The basement membranes of these tissues are not invaded; and the foregut and hindgut, hemolymph, musculature, and other tissues remain uninfected. The pathological changes in the ventriculus or midgut of infected bees have been the subject of a special

study by Hertig (1923). This author describes the following changes: When the cells of the epithelium are filled with *Nosema* spores, the translucent reddish-brown appearance of the ventriculus becomes chalky-white.



A *B*

Fig. 191. Nosema disease of the honeybee, *Apis mellifera* Linn. *A*. Intestines from healthy bees. *B*. Intestines from bees infected with *Nosema apis* Zander. Note the swollen appearance. (*Courtesy of C. E. Burnside.*)

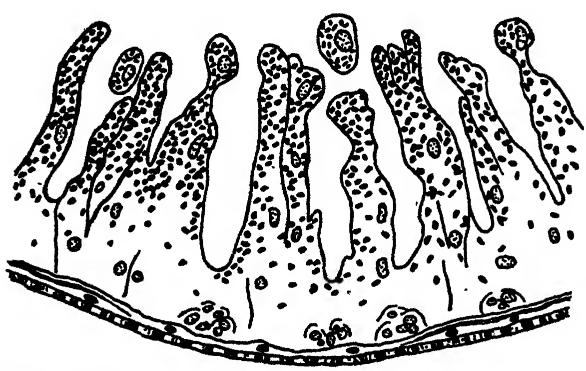


Fig. 192. Diagrammatic longitudinal section of the midgut epithelium of an adult honeybee infected with *Nosema apis* Zander. The numerous dark elements represent stained spores of the parasite. (*Redrawn from White, 1918.*)

The cytoplasm of the cells is largely replaced by the parasites, but the nuclei and cell membranes appear uninjured, although the latter are probably more easily ruptured. There appears to be a tendency toward increased proliferation of the epithelial cells, with a consequent thickening

of the epithelium. As the infection advances, the formation of the striated border and the peritrophic membrane is hindered. Hertig, along with others, assumes that accompanying these pathological changes there is some derangement of the digestive processes, which leads to the malnutrition and hence the weakening and ultimate death of the host.

Some workers have noticed that the gut lumen of *Nosema*-infected bees frequently contains a much larger number of bacteria than that usually present in healthy bees. Whether or not these bacteria play a secondary role in the disease has not been determined, but it is certain that they are not the primary cause of the affliction.

The Causative Agent. The possibility that fungi, bacteria, viruses, or nematodes may be the causative agents of the disease we are discussing has been disposed of adequately. Today there is no doubt that the infectious agent involved in nosema disease is the protozoan *Nosema apis* Zander.

The honeybee, *Apis mellifera* Linn., becomes infected by ingesting the spores of the parasite. Within the midgut of the bee the spore germinates and the binucleated sporoplasm emerges from the spore membrane. The two nuclei soon fuse together and the parasite, now called a "planont," creeps slowly over, and sometimes between, the epithelial cells and ultimately penetrates them. More than one planont may enter a single host cell. Division of the amoebula may occur while it is free in the lumen of the gut, but most of the multiplication occurs within the epithelial cells of the host. Once in its intracellular location, the parasite is less active and, after certain nuclear changes, becomes the form known as a "meront" or "schizont." The meront increases in size and then multiplies by binary fission, multiple binary fission, and delayed multiple division. The meronts become sporonts and undergo sporogony, becoming sporoblasts and eventually spores. Each sporont develops into a single spore. The spores are liberated from the cell usually through that portion of the cell which is normally shed into the lumen of the gut and which, in the case of an infected cell, carries with it many spores. These are carried on through the alimentary tract of the insect and are eventually eliminated with its excreta. Some workers have claimed that autoinfection occurs, but others strongly deny that a spore can germinate in the same host in which it is formed. This point has not been settled. The entire life cycle of the parasite seems to be completed in 3 or 4 days.

The spore of *Nosema apis* is usually oval in shape. Its average size ranges from 4.4 to 6.4 microns long by 2.1 to 3.4 microns wide. The spore membrane is not a rigid structure, but it is highly refractile although somewhat less so than is that of the spore of *Nosema bombycis*. A vacuole

is present at each end, and the sporoplasm is girdlelike in shape. In living preparations the coiled polar filament is not visible, but it may be seen in stained preparations. Under certain circumstances the extruded filament—the length of which is usually between 250 and 325 microns—may show two general regions, one with large undulations and the other with small undulations. There are usually 10 to 15 turns in each region, which fact probably indicates the manner in which the filament is coiled within the spore (Kudo, 1921).

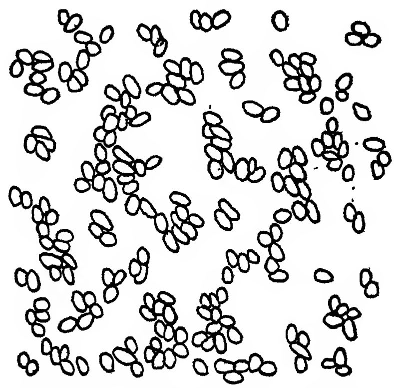


Fig. 193. Diagrammatic representation of a suspension of the spores of *Nosema apis* Zander, the cause of nosema disease in the honeybee, as seen with the higher magnifications of a microscope.

Predisposing Causes. Current belief in some quarters is that *Nosema apis*, in itself, is a microorganism of rather mild invasive properties and probably not a primary cause of the disease. If this is true, then it is important that the factors that predispose bees to the infection be considered.

Phillips is of the opinion that the usual condition which increases the susceptibility of bees to infection is the accumulation of feces in the bees during the winter. Furthermore the protozoa are rarely found in numbers unless the food of the bee contains a rather high percentage of dextrins. Other than this, the kind of food ingested by the insect does not appear to be a very important factor in the disease. Colonies that receive proper care during the winter may still show a few organisms in their digestive tracts, but there is no appreciable death or weakening of bees if they receive proper care.

Other predisposing causes may pertain to age, sex, race, climate, and season. Location of the hive may be important, since, according to Farrar (1947), colonies situated in full sunlight suffer less from the disease than do those in the shade. Sunlight stimulates the flight of bees, and those weakened by nosema disease are less likely to return to the hive.

Adult bees of all ages are susceptible to infection by *Nosema apis*. In nature the youngest bees always are, and old shiny bees usually are, free from infection. White explains the absence of the parasite in the youngest bees by the assumption that they have not as yet been infected by ingesting the spores. The old shiny bees are probably those which have escaped infection or possibly have been lightly infected at

one time and subsequently recovered. The brood does not appear to be susceptible to infection. Even in heavily infected colonies the larvae and pupae apparently remain healthy.

Drone and queen bees are susceptible, but the infection is found most often in the workers, of which frequently 10 or 20 per cent or more may be infected in any one colony. Only occasionally are drones and queens found infected in nature. The weakness of the colony usually results from infection not of the queen or drones but of the workers. So far no significant difference in susceptibility has been found in the different races of bees. Such differences may exist, however.

The presence of nosema disease in a region does not appear to depend directly upon the general climatic conditions present, but it does appear to be related to the season of the year. According to White, it is possible that the climate of a particular region may affect somewhat the occurrence and the course of the disease in that locality. The disease has been reported from widely distant parts of the world under different general climatic conditions. In addition to the United States, it has been observed in Canada, Brazil, Australia, England, Germany, Switzerland, and probably other countries.

The disease may be found in apiaries at all seasons of the year; the incidence is greatest, however, in the spring of the year. In the United States, White (1919) found it to be most prevalent during April and May. Butler (1945) found the incidence in England and Wales to be highest during April, May, and June. The lowest incidence was during November and December. Burnside and Revell (1948) point out that the seasonal rise and fall of nosema disease coincides with seasonal changes in the weather. The average temperature of broodless bees in winter is well below the most favorable temperature (88°F.) for the development of nosema disease. In the spring the bees are likely to be exposed to temperatures highly favorable for the disease. It is during this period that the infection usually spreads most rapidly. By the time continuous warm summer weather has arrived the temperature ordinarily is above the optimum, and the infection subsides. Burnside and Revell found that the maximum temperature for the development of the disease lies between 98 and 99°F., and the minimum between 51 and 57°F. Development is retarded at brood-rearing temperatures (93 to 95°F.) and at temperatures of broodless bees in winter. Diseased bees recover from the infection when held at 99°F. for 14 days. This last observation more or less confirms that of Lotmar (1944) that infected bees recover when kept at 98.6°F. for 10 days.

Transmission of *Nosema apis*. The only manner in which bees become infected with *Nosema apis* is through the digestive tract of the insect.

Accordingly, transmission can be effected only by the ingestion of food, water, or other materials contaminated with the parasite. This ingestion of the parasite may occur during the bees' regular feeding time or when they clean their own or their associate's body, or during the cleaning operations within the hive. Perhaps the greatest source of infection is through contaminated food and water supplies. A stagnant or sluggish pool of water near the apiary into which dead bees or the excrement of infected bees can fall may serve as a source of infection to bees that use the water for drinking purposes. In most instances the food materials and water are contaminated through the fecal droppings of infected bees.

Another source of infection may possibly arise from the habit that bees have of robbing weakened colonies or hives. Transmission by way of flowers, wind, or the hands and tools of the apiarist does not seem to be of sufficient danger to warrant undue concern. No evidence has been gathered which would indicate that other species of insects may be instrumental in spreading the disease, although ants are frequently seen transporting bees dead of *Nosema*-infection. Ants, as well as silkworms and fly maggots, have been inoculated experimentally with *Nosema apis* but were found insusceptible.

The practice of distributing packaged bees for the purpose of replacing winter losses may be a factor in disseminating the microsporidian. It has been shown that nosema disease is largely responsible for the abnormal supersedure of queens in package colonies. Means of reducing the infection in package colonies have been suggested by Farrar (1942, 1947).

Resistance of *Nosema apis*. Pertinent to the control of nosema disease in the honeybee is the resistance of *Nosema apis* to physical and chemical factors. In general, it might be said that the spores of *Nosema apis* do not possess particularly great resistance to most destructive factors in their environment. White (1919) conducted a series of experiments in which he tested the resistance of *Nosema apis* to various influences. In brief, his results were as follows:

Nosema apis suspended in water is destroyed by heating for 10 minutes at about 136°F. (58°C.).

Suspended in honey, *Nosema apis* is destroyed by heating at about 138°F. (59°C.).

Nosema apis drying at room and outdoor temperatures remained virulent for about 2 months, at incubator temperature about 3 weeks, and in a refrigerator about 7½ months.

Nosema apis was destroyed in the presence of fermentive processes in a 20 per cent honey solution in 3 days at incubator temperature and in 9 days at outdoor temperature. In a 10 per cent sugar solution it was destroyed in from 7 to 11 days at room temperature.

Nosema apis resisted putrefactive processes for 5 days at incubator temperature, for 2 weeks at room temperature, and for more than 3 weeks at outdoor temperature.

Nosema apis when dry was destroyed in from 15 to 32 hours by direct exposure to the sun's rays.

Nosema apis suspended in water was destroyed by exposure to the sun's rays in from 37 to 51 hours.

Nosema apis if suspended in honey and exposed to the sun's rays frequently will be destroyed on account of the temperature of the honey which results from the exposure.

Nosema apis remained virulent in honey for from 2 to 4 months at room temperature.

Nosema apis in the bodies of dead bees ceased to be virulent in one week at incubator temperature, in 4 weeks at room temperature, in 6 weeks at outdoor temperature, and in 4 months in a refrigerator.

Nosema apis in the bodies of dead bees lying on the soil ceased to be virulent in from 44 to 71 days.

Nosema apis is readily destroyed by carbolic acid, as 1 per cent aqueous solution destroyed it in less than 10 minutes.

Prognosis and Control. The number of infected bees in a colony may be very small or very large, ranging all the way from less than 1 to 100 per cent of the colony. According to White (1919), between these limits the prognosis in each instance may be different. In general, he found that colonies which in the spring of the year show less than 10 per cent of *Nosema*-infected bees gain in strength and the losses are not apparent. This may be the case also in instances where the infection is somewhat greater than 10 per cent. When the number of infected bees approaches 50 per cent, the colonies become noticeably weakened, and in many instances death of the colony results. In cases where more than 50 per cent of the bees are infected the colony weakens and usually dies. In other words, White concludes that when a colony contains less than 10 per cent of *Nosema*-infected bees the prognosis is excellent; that when it contains more than 10 and less than 50 per cent the prognosis is fair; that when it contains more than 50 per cent the prognosis is unfavorable; and that when the number of *Nosema*-infected bees present approaches 100 per cent the prognosis is especially grave.

The prognosis will vary according to the conditions present: the percentage of infected bees in the colony, the strength of the colony, the season of the year, and the environment of the apiary. The stronger the colony, the more favorable the prognosis. The colony is likely to become weakened in the spring of the year because heavy losses among the workers are not replaced. On the other hand, during active brood-rearing season the bees dying of infection are replaced by young bees and the colony maintains

its strength. Thus the outcome of the disease may to a large extent depend on the time of the year and the activity of the hive.

The length of time an infected worker lives depends upon the season of the year. During the active bee season death usually takes place in 2 to 4 weeks. During the winter months the time of death may be prolonged to 2 or 3 months or more. Whether an individual bee ever recovers once it has been infected is not definitely known. There is some indication that recovery may occur, but such cases are probably rare.

Control of nosema disease of honeybees is of a preventive nature rather than of a therapeutic nature. In the first place, healthy stocks should be removed from the vicinity of diseased bees. The water supply should be protected from contamination and closely supervised. When possible, the water should be changed daily. Some authorities maintain that dead bees and badly diseased colonies ought to be destroyed—preferably by burning. Others feel that such drastic measures are unnecessary. Hives containing infected bees may be charred with a flame, or they may be soaked or washed in formalin or carbolic acid. There is even some question as to whether even this is necessary since, if the equipment is allowed to stand exposed to the sun for several days, the contaminating spores may be destroyed. Rigid cleanliness and good general beekeeping are important safeguards against the disease. Strong, vigorous, healthy, and well-kept colonies are not likely to suffer any great loss from nosema disease. The same applies to package colonies, which should be well supplied with honey and pollen reserves so as to provide for a rapid addition of young bees. The selection and breeding for long-lived bees may also reduce losses from the disease since such bees are able to build up their colonies in the spring even when most of the bees are infected.

Microsporidioses of Destructive Insects

The two microsporidian diseases so far discussed have concerned beneficial insects, the silkworm and the honeybee. Other beneficial insects might have been included, and it should be remembered that diseases among entomophagous and other types of parasitic insects may cause detrimental effects and undesirable consequences by upsetting the balance between the insect parasite and its host. The emphasis placed upon the study of these two diseases is not intended to indicate that microsporidian infections of the less beneficial or harmful insects are not important. The true significance of the microsporidian diseases in the natural control of certain of our destructive insects has not been ascertained with any degree of accuracy. Paillot (1928) has made the statement that protozoa play a much more important part than bacteria in the natural destruction of noxious insects. His basis for this opinion is not explained. It would

appear, however, that such a sweeping conclusion needs further substantiation. Nevertheless the role of protozoa in the destruction of insects in nature must be considerable and is probably much greater than is generally realized. Very likely most of the protozoa concerned in this relationship are Microsporidia.

Of the appreciable number of species of Microsporidia known to infect insects of economic importance, only a few of the better known examples can be given here. Except for the mere reporting of the presence of various Microsporidia in the insects, most of the observations to which we refer have been made in Europe. Examples are the microsporidian infections of the European corn borer, *Pyrausta nubilalis* (Hbn.), and the cabbage butterfly, *Pieris brassicae* (Linn.).

Microsporidiosis of the European Corn Borer. In the areas of the Jura range of mountains, particularly near Bletterans and Chaussin, Paillot (1928) found larvae of the European corn borer, *Pyrausta nubilalis* (Hbn.), infected with a microsporidian which he named *Perezia pyraustae*. (The genus *Perezia* is considered to be differentiated from the genus *Nosema* by the fact that each sporoblast forms two spores instead of one, as is the case in the latter.) In 1927, the number of larvae infected reached 30 to 40 per cent in these focal areas, with lower percentages existing in the outlying and more distant localities. Paillot further reported that the infection existed also in the departments of Tarn and Aude but that infected insects appeared to be completely absent in cornfields south of a line between Lons-le-Saulnier and Louhans. Although rather extensive in its distribution, the infection does not seem to be of much consequence in the natural destruction of the corn borer in France. It may, however, cause a general debilitating effect on the insect, making it more susceptible to other destructive agencies and decreasing its reproductive capacity.

Perezia pyraustae Pail. may attack all stages of its host. The principal seat of the infection is the Malpighian tubes, which become swollen and take on an opaque white appearance. The infected cells of the tubes contain the spores of the parasite, and, according to Paillot, they may be hypertrophied. (The last named characteristic might indicate its closer relationship to the genus *Glugea*.) When the organisms are numerous, the ciliated epithelium of the tubes is largely destroyed.

The mechanism of infection and the life cycle of the parasite are essentially similar to those of other Microsporidia that attack insects. After the corn borer ingests the spore, the sporoplasm emerges and by amoeboid movements proceeds to the Malpighian tubes; sometimes the spores pass directly into the tubes or into the silk glands before germinating. During schizogony the parasite may divide into small chains of binuclear cells, or multinuclear elements may be formed. The small chains usually consist

of two cells each, while the multinuclear elements contain a maximum of four nuclei. The development of the parasite is considerably modified when the host cell becomes filled with the protozoan and the living conditions within the cell become less favorable. Under such conditions the parasite becomes more oblong in shape, the protoplasm develops vacuoles, and certain nuclear changes occur. The cells containing four nuclei are the sporoblasts which, after further division, give rise to spores. Each sporoblast forms two spores, which are of a size somewhat smaller

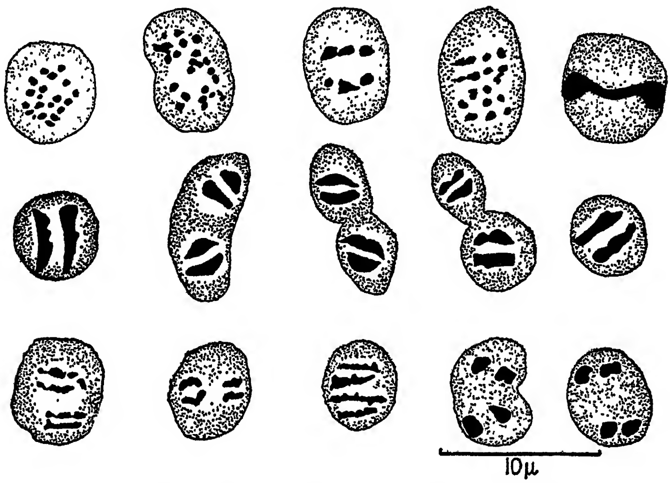


Fig. 194. *Perezia pyraustae* Pail. Stages of schizogenesis; stained with Giemsa's solution. (*Redrawn from Paillot, 1928.*)

than those of *Nosema bombycis*, the cause of pebrine in the silkworm. The spores may differ slightly in size according to the individual host; sometimes double spores and oversized spores are formed.

Transmission of *Perezia pyraustae* takes place through the ingestion of food and other material contaminated by the spores and through the transfer of the spores through the egg to the succeeding generation. An infected larva ordinarily completes its metamorphosis in the usual length of time, retaining the parasite until it leaves the ovipositing female in one of her eggs.

It is not unlikely that the microsporidian described by Kotlån (1928) as *Nosema pyraustae* is the same species as Paillot's *Perezia pyraustae*. Sufficient information to ascertain this possibility has not been furnished. *Nosema pyraustae* is described as occurring on the surface and in the interior of the muscles of the corn borer where it is located in groups of ten to several hundred. Apparently they are present also in other organs of the insect.

Microsporidioses of the Cabbage Butterfly of Europe. At least four different species of microsporidia have been reported as parasites of *Pieris brassicae* (Linn.), the cabbage butterfly of Europe. These are *Perezia mesnili, Perezia legeri, Perezia pieris,* and *Thelohania mesnili,* all discovered and described by Paillot (1918*a,b*; 1924*a,b*).

Perezia mesnili Pail., named after F. Mesnil, was observed by Paillot in 1917 during an invasion by *Pieris brassicae* (Linn.) in the region of Lyon, France. The infection apparently was not very widespread, since, in addition to the area about Lyon, Paillot found it present only in the region of Sathonay-Rillieux, and in both these areas only a small percentage of the insects were parasitized. It was not present in the caterpillars around Saint-Genis-Laval. The microsporidian parasitizes primarily the Malpighian tubes and the silk glands of the insect. Two types of schizogony occur: binary division and multiple division. The spores are elongated and ovoid; their size is variable but averages 3 to 4 microns in length by 1.5 to 2.0 microns in width. The extruded filament appears to be 18 to 20 microns in length. During the same caterpillar infestation, Paillot observed a second species of microsporidian, *Perezia legeri* Pail. (named after L. Léger), which parasitizes the insect's adipose tissue and certain "giant cells" of the blood. Occasionally a generalized infection occurs throughout the body. The giant cells in the blood may reach a size of 150 microns in diameter. On close inspection they are visible to the naked eye and appear as small white granules in suspension in the blood. When filled with spores they resemble floating cysts. They probably originate from the ordinary elements of the blood since all the intermediary stages between these elements and the giant cells are discernible in the blood of individual caterpillars. Multiplication is by binary and multiple fission. The spores are elongated and oval, and from 4 to 5 microns long. The filament, extruded from the rounded end of the spore, measures 30 to 40 microns long.

In 1923 Paillot encountered, along with the two species just mentioned, two additional species of microsporidia in cabbage-butterfly larvae in the region of Lyon. One of these, *Perezia pieris* Pail., is similar to *Perezia mesnili* Pail. but does not multiply in quite the same manner as does the latter species. It is found in the Malpighian tubes, the silk-producing glands, and in the intestinal tube of the caterpillars. The other species, *Thelohania mesnili* Pail., is characterized as having an eight-spore pansporoblast. It is a rare species, having been encountered only twice in the cabbage butterfly. The parasitized larvae are very difficult to distinguish from healthy ones. The adipose tissue, which is the principal seat of infection, appears to have small white tumors. Most of the cells become greatly hypertrophied and are destroyed. Those which are not

destroyed and in which the nuclei remain more or less intact usually contain several parasites.

On the basis of his work on these four species of microsporidia, Paillot concluded that the artificial transmission of certain microsporidia to insects via the alimentary tract is not often possible. He believed this to be due to the high specificity of certain of the parasites for particular tissues of the host. For those species which cannot develop there, the walls of the alimentary tract constitute a barrier that keeps the parasite from reaching those tissues in which it can grow. Thus Paillot was unable to infect the cabbage butterfly via the oral route with either *Perezia legeri* or *Thelohania mesnili*, which parasitize the adipose tissue. On the other hand, when a microsporidian, such as *Perezia pieris* or *Perezia mesnili*, is able to grow and multiply in the cells of the intestinal wall, infection is easily accomplished even with small doses of spores. In neither of these cases of artificial infection, however, does the disease prove fatal for the insects, which seem quite capable of spinning cocoons and completing their development.

For those cases in which infection by way of the digestive route is impossible, two hypotheses have been advanced to help explain the natural transmission of the parasite from one individual insect to another: (1) transovarial transmission in which the parasite passes from the ovary of the infected insect to the developing egg and thence to the newly formed larva; (2) transmission through the intermediation of insect parasites, such as species of *Apanteles*. In the case of the microsporidian diseases of the cabbage butterfly, Paillot always found *Apanteles* larvae in the body cavity of infected larvae. This hypothesis is supported by the fact that in those years in which the population of *Apanteles* is great, considerably more caterpillars are infected with *Perezia legeri* than in those years when there are only a few *Apanteles*. Additional evidence that parasitic insects may be important in the transmission of protozoan infections is provided by the case of the hymenopterous parasite *Microbracon hebetor* (Say) which, by means of its ovipositor, apparently can transmit *Thelohania ephestiae* Mattes from one larva of the Mediterranean flour moth (*Ephestia kühniella* Zeller) to the other. According to Payne (1933), this assumption is based on the following evidence: The disease cannot be transmitted by mouth. Healthy larvae are not infected when kept in the same culture dish with diseased larvae. The disease follows attack by the parasitic insects. The first point of infection is in the ganglion pierced by the parasitic wasp. Later the protozoan is found in both the nervous system and in the fat body.

Microsporidioses of Other Pests of Agricultural Importance. Several other microsporidian infections of agricultural pests have been observed.

In only a few instances, however, has the actual significance of the parasite as a factor in the natural control of the insect been determined. Undoubtedly more species of microsporidia remain to be discovered in destructive insects. Examples of such parasitization include the following, most of which have been reported from outside the United States.

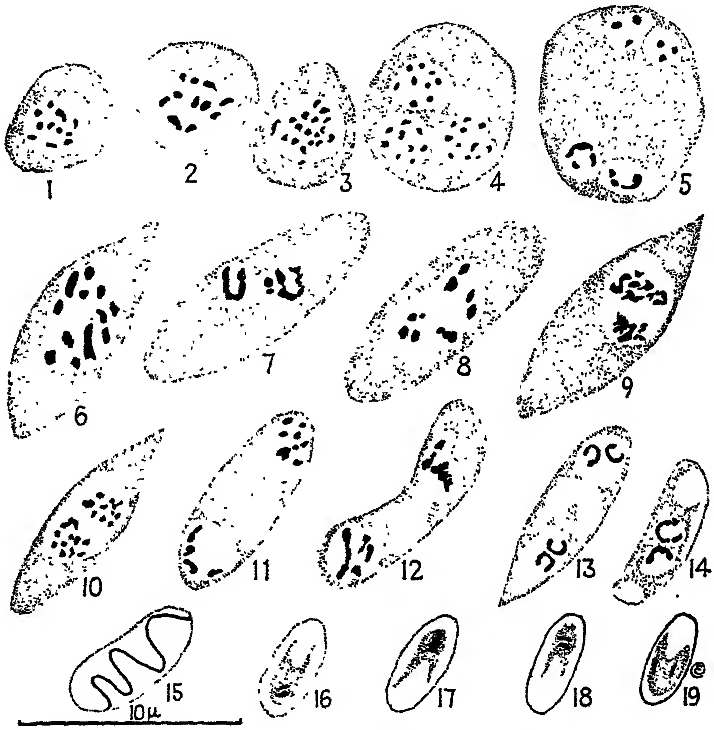


Fig. 195. Various stages in the life history of *Nosema carpocapsae* Pail. as it occurs in the larva of the codling moth. 1–5. Schizogony. 6–13. Sporogeny. 14–19. Spores. (*Redrawn from Paillot, 1939.*)

Nosema carpocapsae was discovered by Paillot (1939) in larvae of the codling moth (*Carpocapsa*) in the region of Saint-Genis-Laval, France. In 1936 the number of larvae parasitized was very small; in 1937 the proportion of infected larvae increased considerably—to almost 40 per cent. Paillot found the same disease to be present also in other regions of the Rhône, particularly in the valley of the Saône at Saint Romain in Mont-d'Or. In general, the microsporidian has not been observed to be an important factor in the natural destruction of the codling moth. The parasite resembles those of the genus *Perezia,* except that some of the sporoblasts are transformed directly into spores without passing through

the intermediate pansporoblast stage. It was for this reason that Paillot placed the organism in the genus *Nosema*. The spores are ovoid in shape and measure approximately 2 by 4 microns in size. *Nosema carpocapsae* Pail. may parasitize most of the cells of the host; but the most frequently affected are those of the silk glands, Malpighian tubes, adipose tissue, muscles, and the oenocytes. The spores are also found in the pericardial cells, the epidermal cells, the cells at the base of the hairs, the genital capsule, and the epithelial cells of the posterior midgut. The latter cells are often discharged into the lumen of the gut, where they allow the contained spores to escape. The infected cell shows no important histopathological changes; the nucleus retains its normal appearance, although the cytoplasm becomes vacuolated and somewhat hypertrophied. A remarkable synchronization exists between the development of the parasite and that of its host. For instance, during the insect's diapause, the parasites undergo no multiplication and occur only in the spore stage.

Nosema heliotidis Lutz & Spl. was described in 1904 by Lutz and Splendore from the corn earworm, *Heliothis armigera* (Hbn.), in Brazil. The microsporidian was named after the generic name of the insect, and the omission of the second "h" was probably unintentional. The spores are more or less elongated and oval; their length is 2.5 to 5.5 microns, their breadth is 1.7 to 2.0 microns.

In France, the weevil *Otiorhynchus fuscipes* Oliv. is attacked by *Nosema longifilum* Hesse, which parasitizes the adipose tissue and forms cysts in the abdominal cavity of the insect. The host tissue reacts against the infection by developing a capsule of connective tissue around the cyst. Hesse (1904) describes the microsporidian as having two kinds of spores. The type most frequently seen is oval in shape and from 4 to 5 microns long and 3 microns wide. The filament has a length of 85 to 90 microns. Some of these spores have a large vacuole at one end. The second type of spore is elliptical and has an approximate size of 6 by 4 microns. These spores always appear to be empty.

In the summer of 1929, Chorine (1930) found 6 to 7 per cent of the 600 larvae of the small tortoise-shell butterfly, *Vanessa urticae* (Linn.), submitted to him from Yugoslavia, to be infected with a microsporidian which he named *Thelohania vanessae*. The diseased larvae are sluggish in movement and soon cease feeding, about a day after which they suffer a severe diarrhea and vomit large quantities of a bright green transparent liquid that oozes from the mouth. None of these discharges contain the parasite. The following day the caterpillars hang by their prolegs and die. The duration of the disease is usually 10 or 11 days. The blood of the dying insects contains the microsporidia, which are also present in the adipose tissue and in the covering of the genital glands. The fat cells,

which are usually packed with the parasite, are almost destroyed except that the nuclei are not invaded although they are hypertrophied and become granular in appearance. The silk glands, muscles, and Malpighian tubes remain intact. The parasites are occasionally found in the intestinal cells. The ovoid spores vary from 4.2 to 6 microns in length by 3 to 4 microns in width. The extruded filament varies from 60 to 120 microns in length. Transmission of the disease occurs through the egg and possibly through the agency of insect parasites such as *Apanteles,* although Chorine considers the latter type of transmission to be purely accidental. The microsporidian appears to be specific for caterpillars of *Vanessa.* In the blood of the wax moth, *Galleria mellonella* (Linn.), the spores liberate the amoebulae, which are then phagocytosed. *Carausius morosus* Brunn. is completely refractory to the protozoan.

Plistophora schubergi is the name given by Zwölfer (1927) to a microsporidian parasite of the gypsy moth, *Porthetria dispar* (Linn.), and the brown-tail moth, *Nygmia phaeorrhoea* (Donov.). Zwölfer considered the infections produced by this protozoan to be of considerable agricultural significance, even more so than the polyhedral disease of the gypsy moth, especially when conditions were such as to promote its spread. As with most of the protozoan diseases of insects, this infection was not explosive in nature but was rather of an incipient character, and one in which the destruction of the insects increased gradually. Infections of 70, 84, and 94 per cent of caterpillars have been observed.

Microsporidioses of Mosquitoes. Among the insects that are susceptible to infection by microsporidia, mosquitoes are prominent. At one time it was thought that microsporidia might serve as an antimosquito measure. This idea has now been abandoned not only because of the efficiency of modern larvicides but also because areas containing large numbers of infected individuals continue to produce mosquitoes in their usual abundance. Fatal infections do occur, however, and decreases in the number of mosquitoes as a result of infection undoubtedly do take place, but these decreases are rarely significant from the standpoint of obtaining adequate control.

A considerable number of species of microsporidia have been seen in mosquitoes, but they have not been described systematically or named by their discoverers. They frequently have been identified as to genus but not as to species. Of the several named species concerned, approximately 4 are of the genus *Nosema,* 12 of the genus *Thelohania,* and at least 1 each of the genera *Plistophora* and *Stempellia.* The microsporidia are usually specific as to the genus of the host, and most species of mosquitoes so far found to be susceptible appear to belong to the genera *Anopheles, Aëdes,* and *Culex.*

The life cycles and other characteristics of the microsporidia infecting mosquitoes are typical of most species within the genus concerned. Furthermore the size and shape of the spores are typical of those of most microsporidian spores. Thus *Thelohania legeri* Hesse, which parasitizes more than a dozen species of *Anopheles*, has ovoid spores with rounded ends and is 4.7 to 6 microns long by 3 to 4 microns wide. *Stempellia magna* Kudo, parasitic in species of *Culex*, has an elongated pyriform spore measuring 12.5 to 16.5 microns long by 4 to 4.6 microns broad.

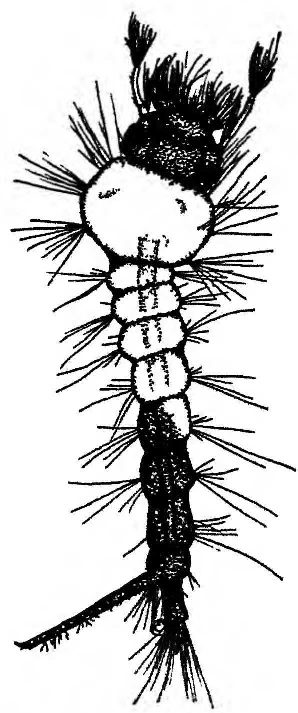


Fig. 196. Dorsal view of a larva of *Culex apicalis* Adams infected by *Thelohania opacita* Kudo, showing the characteristic white opacity of the entire thorax and the anterior four segments of the abdomen. (*Redrawn from Kudo, 1921.*)

The symptoms of the different microsporidian infections in mosquito larvae vary somewhat, but in general they are fairly uniform. Numerous observations in this regard have been made by the protozoologist Kudo. Infected larvae are usually sluggish in movement or almost entirely inactive, and they may be stunted or diminished in size. Sometimes there is a deformity of the body which may cause peculiar or abnormal body movements. Certain segments may be greatly distended. Almost always the body exhibits a certain degree of opacity caused by the presence of large numbers of microsporidian spores. The opacity may be from slight to marked in density. The larvae may fail to pupate and then die or, if the infection is light, the insect may complete its development. Infection apparently takes place by way of the digestive tract through the ingestion of spores. Since many species feed on the dead bodies of their fellows, infection may be contracted in this manner. Surface feeders that live in running water (*Anopheles*) appear to be less frequently infected than do species (of *Culex*) that live in stagnant water and feed at the bottom.

The internal pathology is usually most noticeable in the adipose tissue. The nuclei of the fat cells hypertrophy, and the cytoplasm becomes distended with spores. Fat cells that are heavily infected may rupture, liberating the parasites into the general body cavity of the insect. Most other tissues remain free of the infection. The muscle cells, however,

may become degenerated and thus weaken the larva. Occasionally a phagocytosed spore is seen in a blood cell of its host. In the case of some infections the parasitization may be of a more general nature. For example, *Plistophora stegomyiae* (M., S., & S.) is known to parasitize the alimentary tract, coelom, Malpighian tubes, ovary, ova, muscles, ganglia, tracheal epithelium, and possibly other tissues of adult *Aëdes aegypti* (Linn.).

Two other rather closely related groups of Diptera known to serve as hosts to a number of microsporidia are those belonging to the families Simuliidae and Chironomidae. Named species of microsporidia have been described from about 10 species of simuliid hosts; unidentified species also have been seen frequently in these insects. It was from an unidentified *Simulium* larva that Weiser (1946) described the interesting new genus *Caudospora*. The spores of these microsporidia are flattened and characteristically have a long caudal prolongation. With regard to the microsporidian infections of Chironomidae (and Ceratopogonidae) larvae, Weiser (1943, 1947) lists 18 species having representatives in the following genera: *Nosema*, *Thelohania*, *Plistophora*, *Cocconema*, *Octosporea*, *Toxoglugea*, *Spiroglugea*, *Bacillidium*, and *Mrazekia*.

OTHER SPOROZOAN INFECTIONS

In addition to those already discussed, there remain to be considered those species of protozoa which belong to the class Sporozoa but whose further systematic allocation is not entirely clear.

Mycetosporidium. Two such species are *Mycetosporidium talpa* L. & H., parasitic in the weevil *Otiorhynchus fuscipes* Oliv., and *Mycetosporidium jacksonae* Tate, parasitic in species of *Sitona* weevils. The first-named species was described in 1905 by Léger and Hesse, who found the parasite confined largely to the intestine of its host. *M. jacksonae* was described by Tate (1940), who observed that the infection in *Sitona* occurred in the Malpighian tubes as well as in the intestinal epithelium. According to this author, the life history comprises vacuolated and compact plasmodia in which arise ovoid or spherical multinucleate bodies resembling coccidian schizonts. Sporulation results in the formation of eight-nucleate biconvex spores within thin-walled sporangia. The spores themselves possess densely staining resistant walls. Other types of multiplication occur. In one case multiplication is in the form of small uninucleate ovoid or fusiform bodies that develop directly from the plasmodia; in another case four to six small uninucleate ovoid or fusiform bodies are formed within each chamber of a multilocular sporangium.

In the early stages of the infection the parasites invade the intestinal wall and are found near the basement membrane. The later stages of plasmodial development and spore formation may be seen especially in

the areas between the nests of replacement cells. In this location the parasites tend to extend toward the lumen of the intestine in broadly conical formations. Tate found that the early stages are usually distinctly intracellular and that young plasmodia were often present in the very immature replacement cells of the intestinal epithelium. The developing plasmodia are carried toward the lumen of the intestine by the growth of the replacement cells and eventually are released into the lumen in the form of compact, multinucleate, schizontlike bodies, or as masses of thick-walled spores. The boundary between the cytoplasm of the host and that of the parasite is often indistinguishable. The nucleus of the parasitized host cell may be considerably distorted by the pressure exerted on it by the growing parasite, and although degenerate, it usually remains distinguishable even in cells almost completely destroyed by the parasite.

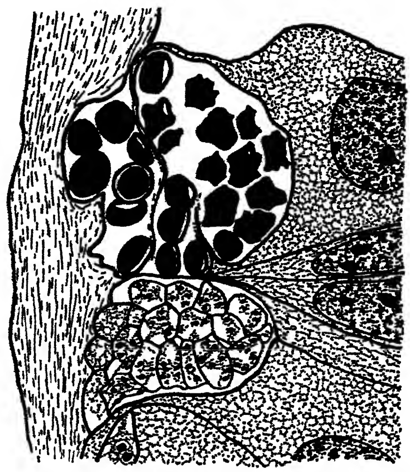


Fig. 197. *Mycetosporidium jacksonae* Tate parasitic in *Sitona* weevils. Part of a transverse section of the gut of *Sitona* showing (above) a sporangium containing mature thick-walled spores; and (below) a multilocular sporangium within each chamber of which the cytoplasm is aggregating around each of the nuclei to form small ovoid, or fusiform, uninucleate bodies. *(Redrawn from Tate, 1940.)*

The histopathology of the Malpighian tubes may be more advanced, especially in cases of heavily infected specimens. In such individuals, masses of spores or large vacuolated plasmodia may completely replace the cells of the tube. Infected cells may bulge into the lumen of the tube and later are shed, along with the contained parasites, into the lumen where they lie free. The same stages of development that occur in intestinal infection are also found in the cells of the Malpighian tubes.

Helicosporidium. Another interesting sporozoan infection is that caused by *Helicosporidium parasiticum* Keilin in larvae of the Diptera *Dasyhelea obscura* Winn. and *Mycetobia pallipes* Meig., and the mite *Hericia hericia* (Robin). This peculiar protozoan was described in England by Keilin in 1921(*a*), and for it Kudo has proposed the order Helicosporidia.

All larval stages of *Dasyhelea obscura* Winn., which lives in the decomposed sap filling the wounds of trees, are susceptible to *H. parasiticum*. Infected larvae usually assume a milky-white opacity that enables one

to distinguish them from normal specimens, which are white but translucent. A similar opacity is present in those larvae infected with a parasitic yeast (*Monosporella unicuspidata* Keilin), but Keilin found the proportion of these larvae to be very small. When examined under the microscope, the entire body cavity of a parasitized larva is seen to be filled with small round corpuscles, 5 or 6 microns in diameter, which occupy all the spaces between the various organs of the body. This infection extends throughout all the segments, including the head. Since most of the parasites occur free in the body cavity, they may be seen to circulate passively throughout the hemocoele as the result of the regular movements of the host and its internal organs. The parasites appear to be most numerous in the posterior segments of the body where solid masses of them occur. The mobility of the posterior end becomes impaired, this portion of the larva becomes more turgid and fragile, and by disrupting the integument large numbers of the parasitic corpuscles escape in the form of a milky-white suspension. Larvae in the early stages of infection are difficult to detect.

Although all stages of the parasite may be found free in the body cavity of the insect, some tissue destruction and pathology are evident. The fat body and nerve ganglia are the tissues most commonly involved, especially in recently acquired infections. When the fat body is attacked it is rapidly destroyed, and the parasites, attached to the fat droplets, escape into the body cavity. When nerve ganglia are parasitized, they become swollen and are reduced to the neurilemma or sheath. Keilin observed that several successive nerve ganglia of the ventral chain may be infected but that the parasites are never found in the nerve commissures.

The characteristics and life cycle of *Helicosporidium parasiticum* are unlike those of any other known sporozoan. The youngest stage is represented by small round trophozoites with a diameter of 2 or 3 microns. After growing slightly, the parasite divides into two cells of equal size. Multiplication continues by schizogony, and the schizonts form a small morula consisting of four or eight merozoites which become free. This cycle may repeat itself. In time, the merozoite resulting from the schizogony increases slightly in size, becomes very basophilic, and produces four cells surrounded by a thin wall or sporocyst. The completely formed spore has a diameter of 5 to 6 microns. Three of the four cells are amoeboid and form the true sporozoites. The fourth cell develops into a peripheral spiral filament surrounding the central cells (see Fig. 198). The sporozoites are liberated when the unrolling of the spiral filament opens the spores inside the dead body of their host. When entirely unrolled the spiral filament is from 60 to 65 microns long and 1 micron thick at its widest part, being pointed at both ends. Transmission of the parasite apparently

occurs via the alimentary tract of the insect. After being swallowed, the sporozoites probably penetrate through the gut wall of the insect into the body cavity, where they begin the schizogonic cycle.

During the course of a year several generations of *Dasyhelea obscura* Winn. occur, all of which are equally susceptible to infection by *H. parasiticum*.

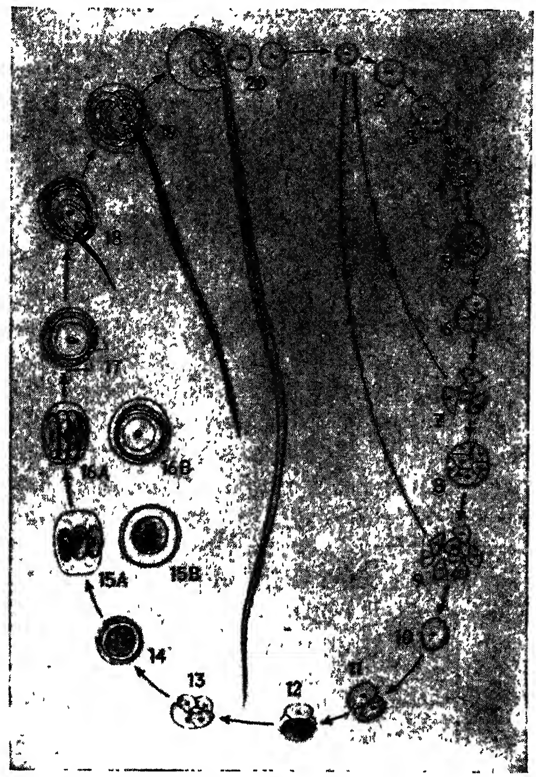


Fig. 198. Diagrammatic representation of the life cycle of *Helicosporidium parasiticum* Keilin. Stages 1–16 occur in the living larva of *Dasyhelea*, but stages 17–20 are found only in the dead body of the host. (*From Keilin, 1921a.*)

The true rate of infection is difficult to determine, however, since this depends upon the condition of the wound of the tree in which the insects live, at the time specimens are collected for examination. According to Keilin, in rainy weather the larvae leave the flooded parts of the tree's wounds and penetrate into the fissures of the tree. In the meantime, the wound is thoroughly washed by the rain and is freed from the collected sap which usually contains dead and dying larvae infected with the parasite. Upon the restoration of normal conditions the wound is once more covered with freshly exuded sap, and the larvae crawl from

their hiding places and again invade the wound. At such times very few infected larvae are found. On the other hand, after the old sap has remained in the wound for a prolonged period, considerably more infected larvae may be collected.

CILIATA

The subphylum Ciliophora consists of two classes, Ciliata and Suctoria. The class Suctoria is represented by exceedingly few species associated with insects, and none of these is distinctly pathogenic for its host in the usual sense of the word. On the other hand, Ciliata, or the ciliates, contains several species that are parasitic in insects.

CILIATE INFECTIONS IN INSECTS

None of the ciliate infections bears a distinctive name; hence, for convenience, they might be considered according to the genus of the parasite concerned. In the general sense, an infection caused by a ciliate may be referred to as a "ciliatosis."

Glaucoma Infection. In 1922 MacArthur reported the presence of a ciliate in the body cavities of living and dead larvae of the mosquito *Culiseta annulata* (Schrank) (= *Theobaldia annulata* Schrank) which he had collected near Blackpool, England. Two years later in France, Treillard and Lwoff (1924) observed, in larvae of *Chironomous plumosus* Burrill, a ciliate which they determined to be *Glaucoma pyriformis* and which apparently is the same species as that reported by MacArthur. In Northern Rhodesia it is apparently a parasite of *Aëdes* (*Finlaya*) *fulgens* Edw. and probably of other culicine mosquitoes (Muspratt, 1947).

Although *G. pyriformis* is definitely pathogenic for certain insects, it does not appear to be an obligate parasite, as is indicated by Wenyon's (1926) success in keeping cultures of it in water, hay infusions, and in the liquid on the surface of agar plates. The ciliate is thought to be acquired by the mosquito through the latter's ingestion of it, after which it penetrates through the intestinal wall of the insect into the body cavity. In infected specimens the parasites may be seen in the cavities of the thorax, abdomen, siphon, and particularly the head, which may be filled with 200 or more ciliates; the antennae may be closely packed with them. The gills are usually free of the parasite. Of particular interest is MacArthur's observation that the destruction of the insect's eyes seems to be a rather constant feature in the infected mosquito larvae. In fact, an examination of the eye condition constitutes one of the first tests of suspected infection. The ciliates have been seen attacking the eyes, during which time the organisms are surrounded by whirling clouds of pigment kept in motion

by their cilia. (This, of course, is not to say that *Glaucoma pyriformis* Ehren. has anything to do with the entirely unrelated disease glaucoma of the human eye.) On the other hand, Muspratt (1947) reports that the parasite which he studied in Africa, and which he considered to be *G. pyriformis*, does not attack the eyes of the host. In very small larvae, the protozoa congregate around the heart.

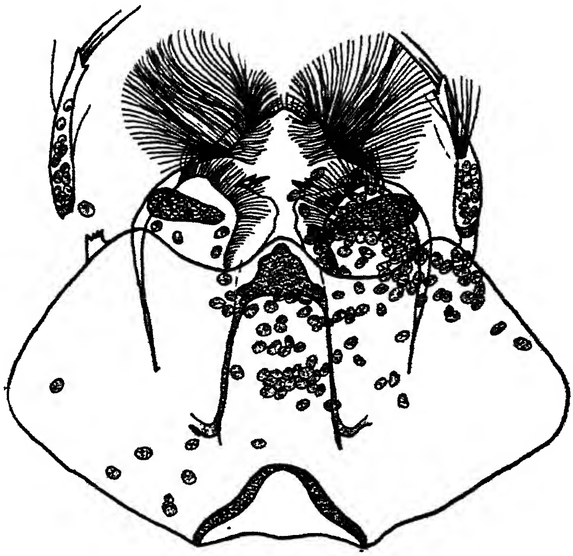


Fig. 199. The head of a larva of *Culiseta annulata* (Schrank) infected with *Glaucoma pyriformis* Ehren. (*Redrawn from MacArthur, 1922.*)

MacArthur described the ciliate from the larva of *Culiseta annulata* (Schrank) partly as follows: The body varies in shape from elongate oval to broad oval and is longitudinally striated. Although there is considerable difference in size, most of the forms vary in length from 25 to 40 microns and in width from 15 to 25 microns. The smallest forms measure 8 or 9 microns in length and the largest about 57 microns in length. There are marked variations in shape; the rapidly swimming forms are relatively long and narrow and are concave ventrally and convex dorsally, while the more slowly moving individuals are relatively shorter and broader, and more or less barrel-shaped. The nucleus is spherical and measures 9 to 12 microns in diameter. The cytostome is placed anterolaterally and is surrounded by circumoral cilia. The endoplasm is granular and usually contains from 1 to 27 food vacuoles, and a contractile vacuole is located posteriorly. When the exoskeleton of an infected larva is ruptured, ciliates rush through the opening and swim about actively tending to

slow down later; many motile ciliates remain inside. The freed protozoa die more rapidly than those remaining within the insect.

The pathogenicity of *G. pyriformis* for insects is further substantiated by the experiments of Lwoff (1924) and by those of Janda and Jírovec (1937). Lwoff inoculated larvae of the wax moth, *Galleria mellonella* (Linn.), with cultures of the ciliate and found that a fatal infection resulted. Janda and Jírovec inoculated bacteria-free cultures of *G. pyriformis* into crustaceans, molluscs, annelids, insects, amphibians, and fish. Only the insects, of which there were 14 species, became infected. In most of these cases the body cavity of the insect became filled with the ciliates within a few days after injection, and of the various tissues, the parasites were found most abundant in the fat bodies. The size of the protozoan appeared to be much greater in the insect than in the cultures. Most of the insects died from the infection in a few days. It is of interest to note that the development of the ciliate infection in the insects depended upon the temperature as well as upon the amount of culture inoculated. As might be expected, development was much slower at lower temperatures (1 to 4°C.) than at higher temperatures (*e.g.*, 26°C.). If an infected insect was held at 32 to 36°C. for ½ to 3 hours, the ciliates apparently were killed and the insect survived.

Lambornella Infection. A ciliate infection similar to the one just discussed was found in 1921 by Lamborn in the Malay States. The host in this instance was the larva of *Aëdes scutellaris* (Walk.) (= *Stegomyia scutellaris* Walk.), and the ciliate was described by Keilin (1921*b*) and named *Lambornella stegomyiae.* Some authors (Wenyon, 1926) believe, since Keilin was unable to make out the details of structure essential for the identification of a ciliate, that the genus *Lambornella* is actually without definition. Furthermore there is a possibility that this parasite is the same as that described by MacArthur from mosquito larvae, which we have already considered under the name *Glaucoma pyriformis.*

Lambornella stegomyiae Keilin is parasitic in all parts of the body cavity of the *Aëdes* larvae and extends especially into the siphon and the tracheal gills which may contain 200 or more individuals. The head and the antennae may also be filled with the parasite. The fat body may be invaded or, in places, completely destroyed. Infected larvae are considerably paler and more opaque than are normal specimens. The ciliates may escape from the larva while it is still alive, either by a rupture in the insect's integument or by the complete separation of the gills. Diseased larvae frequently may be detected by the absence of one or more gills. The infection usually results in the death of the larva, which is unable to pupate and complete its development. Within a day or two after the insect dies, most of the parasites have moved out of the interior.

The protozoan varies in length from 50 to 70 microns and in width from

20 to 30 microns. It is elongately oval to pear-shaped and is uniformly covered with cilia, which are arranged in parallel longitudinal rows. There is a spherical macronucleus and a micronucleus; the latter usually lies in a small peripheral depression of the macronucleus. Reproduction is by simple transverse fission. Hemispherical "cysts," 30 to 40 microns in diameter, have been reported as attached to the external surface of the insect's cuticula.

Infection is probably acquired through the mouth of the insect by virtue of the cysts. There are indications that the cyst has to be ripe before it is infectious; *i.e.*, it is possible that the cysts of *Lambornella* pass through some developmental phase before they become infectious.

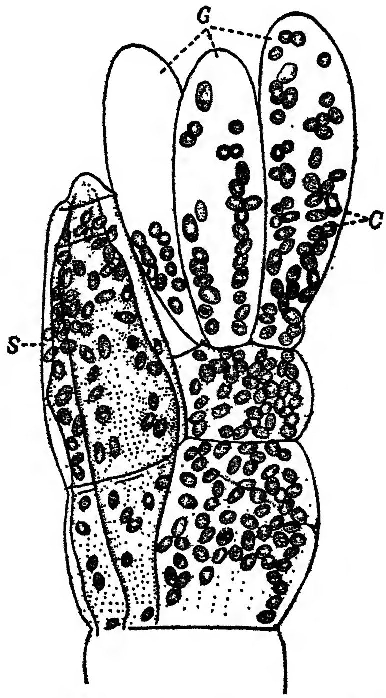


Fig. 200. The ciliate *Lambornella stegomyiae* Keilin parasitizing the posterior end of a larva of *Aëdes scutellaris* (Walk.). *S*, siphon; *G*, gills; *C*, ciliate. (*From Keilin, 1921b.*)

Other Ciliate Infections. Much greater in size than the two ciliates already discussed is the species *Ophryoglena collini* Lich., which Lichtenstein (1921) found parasitizing ephemerid larvae (*Baetis*). This parasite is destructive to the internal tissues, particularly the generative organs, of its host. The ciliate is ovoid in shape and measures 200 to 300 by 120 to 230 microns.

The order Spirotricha contains several species of ciliates that live in the guts of insects. Few of these, however, are actually pathogenic for their hosts. Species of the genera *Balantidium* and *Nyctotherus* are examples. Cockroaches are common hosts.

Mercier and Poisson (1923) have reported the parasitization of a nymph of the hemipteran *Nepa cinerea* Linn. by a species of Colpoda. The ciliate invaded the body of the insect and produced a "tumor" about the size of a pinhead on the lateroventral surface of the metathorax. Numerous ciliates were contained in the tumor, which extended partly inside and partly outside the host's body.

The peritrichous ciliate *Operculariella parasitica* Stammer parasitizes the esophagus of dytiscids in Europe (Stammer, 1948).

Sometimes the relation between a protozoan and its insect host may

appear to be purely a commensal one when in fact some detriment is caused the insect. At least there are instances in which the insect would be better off without the protozoan than it is with it. For example, Elson (1933) observed that a species of *Epistylis*, a stalked, peritrichous ciliate, clings to the external surface of various parts of the body of the hydrophylid beetle, *Tropisternus californicus* (Lec.), and causes the insect considerable difficulty in raising its elytra to obtain air as it comes to the surface of the water to replenish its oxygen supply. In mosquito larvae vorticella ciliates may occur as secondary invaders to bacteria (Jettmar, 1947).

For the names of those Hexapoda on which peritrichous ciliates have been observed, the interested reader is referred to a list compiled by Nenninger (1948).

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CHAPTER 13

NEMATODE INFECTIONS

The preceding chapters have dealt with parasites consisting of but a single cell or of groups of independent cells. We turn now to a consideration of those small multicellular or metazoan animals known as "nematodes" or "nemas," or as Shakespeare had King Richard II say, "Let's talk . . . of worms." Some of these animals are well known for the diseases they cause in man; *e.g.*, *Wuchereria bancrofti* (Cobbold), transmitted by mosquitoes, is the cause of human filariasis, and *Necator americanus* (Stiles) is the cause of hookworm infection. Plants are also attacked, the well-known sugar-beet nematode being an example, and such infestations are of considerable economic and agricultural importance. Many, however, parasitize and destroy insects, and it is these with which we shall be concerned here.

The phylum Nemathelminthes (roundworms) includes three classes: Nematoda, Nematomorpha, and Acanthocephala. The members of these classes have the following characteristics in common: (1) the body is elongate, unsegmented, and usually threadlike; (2) it is enclosed in a hardened cuticula; (3) it has no appendages, cilia, vascular system, or special respiratory organs.

Those Nemathelminthes for which insects serve as primary hosts are included in the classes Nematoda and Nematomorpha. In their general external appearance, all the nematodes are very much alike. Rarely is there any distinct variation in the diameter of the individual nema, although the body tapers toward one or both ends. The anterior end is slightly more blunt than is the posterior end. The smaller living nematodes are more or less transparent, but the larger species are usually opaque. The movement of most Nematoda is fairly characteristic. In a clear fluid medium, the worms undergo rather vigorous coiling and twisting to the right and to the left without making much progress. If solid particles are present in the fluid, however, the organism changes its movement to one of serpentine character, winding in and out of the particles.

Of the class Nematomorpha, members of the subclass Gordiacea (hairworms) are parasites of insects. They differ from the Nematoda in being larger, as a rule, and more uniformly cylindrical, with bluntly rounded ends, and having a faintly colored exterior.

Acanthocephala are known as thorn-headed worms. They are highly specialized for parasitic life and have no free-living stage and no trace of an alimentary tract at any stage of their development. Some of them spend the larval stage within an insect host and the adult stage in mammals.

In general, there are three main stages in the developmental cycle of nematodes: eggs, larvae (including four growth stages), and adults. The eggs are extremely minute—being microscopic in size. The developing embryos are usually discharged as eggs, but in a few species they hatch within the uterus of the female worm and are brought forth viviparously. The embryo may require weeks or months for its growth and may wait within its shell for indefinite periods before it is passively introduced into a new host. Upon hatching, the larvae have the main morphological characteristics of adult nematodes except that the reproductive system and the secondary sexual characters are lacking. Usually the young larvae spend a short period as free-living organisms, frequently in an aquatic environment of water or mud. The larvae of nearly all nematodes undergo four molts before becoming adults. These molts may take place in the egg, during the worm's free existence, in the tissues of an intermediate host, or in the definitive host. Most nematodes are bisexual, the females producing fertile eggs after copulation. Males are frequently less common than females, though usually both sexes are plentiful. The males reach maturity a little sooner and do not live quite so long as do females; this sometimes gives the impression that only females are present.

The cultivation of nematodes on artificial or synthetic media has been attained in a few instances.

Insect Hosts Affected. Within any order of insects, the number of known specific hosts to any particular group of organisms frequently depends simply on the degree to which the insects in any one order have been studied from this viewpoint. Therefore, merely citing the number of nematodes described from the various orders of insects would not necessarily indicate the true range of parasitism as it occurs in nature.

In 1928 van Zwaluwenburg presented a list showing the number of insect species involved as hosts to nematodes according to the various insect orders. Out of a total of 759 insect species (in 16 orders) with which nematodes were then known to be associated, the majority (268) were in the order Lepidoptera. As van Zwaluwenburg suggests, this is because of the intensive work of Schultz (1900), who gave special attention to this order. Next in number of host species is the order Coleoptera with 172 species, then Orthoptera with 116 species, and Diptera with 96 species. Lesser and decreasing numbers were of the orders Hymenoptera, Isoptera,

Hemiptera, Odonata, Siphonaptera, and others. The total number of insect hosts of nematodes known today can only be estimated. La Rivers (1949) has listed 97 insect hosts (some new and some repetitions) which have been reported in the literature up to 1946 and since Zwaluwenburg's paper appeared.

As to the number of species of nematodes associated with insects, only speculative estimates can be suggested. The number of species already reported from this habitat appears to be in excess of 1,000 species.

Relations to Host. The biological relations between nematodes and insects vary all the way from those of mere fortuitous association to those of strict and destructive parasitism. Van Zwaluwenburg (1928) grouped the entomophilic roundworms into five classifications: primary parasitism, secondary parasitism, internal mechanical association, external mechanical association, and commensalism. In the last-named category were placed those nematodes which live within the burrows or nests of beetles, termites, and ants, and which feed upon the frass and debris that accumulates in the nests. Some authors (*e.g.*, Filipjev and Stekhoven, 1941) simply separate the insect nematodes into two large groups: those living within the alimentary tracts of insects, and those inhabiting the body cavity of insects. Since there are so many variations and gradations in the types and degrees of "parasitic" associations between insects and nematodes, it is difficult to draw a true line between them.

Perhaps one of the most convenient groupings to follow is that used by Christie (1941), who divided the nematodes associated with invertebrates into three groups: (1) Those nematodes which live in the alimentary tract of the invertebrate and which are not included in the next group. In most cases the life cycles are simple. (2) Those nematodes which are more or less closely related to free-living species and which often have a combination of saprophagous and parasitic habits. They may live and reproduce in the cadaver of the host, which may or may not have been killed by the parasites. Others of this group may pass through one or more free-living generations which alternate with one or more parasitic generations. Christie designates these organisms as novitious parasites and semiparasites. (3) Those nematodes which parasitize the body cavity or the tissues of their host. These worms are highly specialized, obligately parasitic, and at the most spend only a transitory period in the alimentary tract of the invertebrate. Of these three groups the insect pathologist is perhaps most interested in the last, but the other two have members that are also important from a pathological standpoint as well as from the standpoint of their role in biological control.

The remainder of this chapter will be devoted to a brief consideration of a few important members of the three groups just designated.

NEMATODE PARASITES OF THE INSECT GUT

As it does for many microorganisms, the alimentary tract of an insect affords ideal living conditions for certain nematodes. A great majority of those nematodes which have this habitat are grouped in Filipjev's (1934) category Oxyurata. According to Christie (1941), nematodes belonging to the families Thelastomatidae and Rhigonematidae and to the subfamily Ransomnematinea are parasites of the alimentary tract of animals, and there is an occasional species of the family Diplogasteridae that has acquired this mode of life. Christie also tells us that the literature contains descriptions of between 60 and 70 species of thelastomatids parasitic in the gut of insects and myriapods. Most of these descriptions are of a taxonomic character only, and very little information is available as to the biological relationships existing between them and their hosts. So it is with the other groups. In general, very little apparent harm is caused the host by the parasites, which have simple life cycles and rarely invade vital tissues of the insect. In most cases, the eggs of the nematode do not hatch in the gut of the insect but pass out with the feces. Once outside the host they undergo further development until they reach an infective stage. Infection of a fresh host occurs when these infective eggs are ingested by the insect along with its food.

The biology of this group of nematodes has been only meagerly studied, but enough information has been gathered to indicate that the oxyurids inhabit the guts of those insects which have well-developed digestive systems. Such digestive systems afford almost perfect digestion of the food, combined with a slow passage of the food through the gut, a comparatively long stasis of the fecal pellets in the rectum, and a rich bacterial flora (Filipjev and Stekhoven, 1941). Accordingly, these nematodes are rare in the caterpillars of Lepidoptera and in species of Locustidae—groups in which the food passes rapidly through the alimentary tract and is eliminated only partly digested. Insects that do harbor these nematodes include species of Blattidae, Scarabaeidae, Passalidae, Hydrophilidae, and others. Many of the oxyurid nematodes live in the rectum of the insect, ingesting the rectal contents and absorbing mostly those food substances which are of little value to the host.

Examples. Since very little harm is caused the host and practically no pathology results from the infestation, a detailed discussion of these nematodes will not be necessary here. Two species, however, may be cited as examples of the group.

Cephalobium microbivorum Cobb, a diplogastrid, lives in the gut of the black field cricket, *Gryllus assimilis* (Fabr.). An individual insect may harbor up to 30 or more nemas, the eggs of which pass out of the host with

the feces. The insect apparently acquires the "infection" by way of the mouth. Infected crickets have been collected in Virginia and in Kansas.

Leidynema appendiculatum (Leidy), a thelastomatid, is a parasite of the cockroaches *Blatta orientalis* (Linn.) and *Periplaneta americana* (Linn.) in Kansas and probably elsewhere. The egg of this nematode passes out with the feces of the insect and undergoes a short period of development, forming a small tadpolelike larva. Still within the egg the larva, at first motile, becomes inactive and forms the infective stage in about 3 to 7 days. The egg completes its life cycle when it is ingested by the insect and hatches in the hind portion of the midgut where the worm matures. No evidence of harm to the host insect has been obtained.

Fig. 201. *Leidynema appendiculatum* (Leidy), a parasite of cockroaches. Adult, female. (*Redrawn from Dobrovolny and Ackert, 1934.*)

At this point the fact might be mentioned that some nematodes (*Spirurata* and *Filariata*) are parasites of vertebrates but have insects as intermediate hosts. In certain cases the insect serves as a transmitting agent for the larval nematodes. The insects, depending on the species, may acquire the nemas by the sucking of infectious blood or by feeding upon or contacting the droppings or refuse of animals. The nematologist can usually quite readily differentiate these nematodes from those which parasitize or are specifically associated with insects.

NEMATODES SEMIPARASITIC IN INSECTS

Neoaplectana glaseri Steiner in the Japanese Beetle

In May, 1929, while digging for grubs of the Japanese beetle, *Popillia japonica* Newm., near Haddonfield, New Jersey, Glaser and Fox (1930) found a number of dead fully grown larvae, which upon dissection were observed to be infected with nematodes. Later the same season parasitized pupae and adults were collected. Specimens of the nematode were sent to Steiner (1929), who named it *Neoaplectana glaseri*, commenting that it is probable that the worm has for an original host some native insect or insects and has only recently adapted itself to the Japanese beetle. Steiner placed the nematode in the family Oxyuridae, but it has since been placed in the family Steinernematidae by Christie (and Anguillulidae by Filipjev). Subsequent to its discovery, recoveries of the parasite from the original Haddonfield area have been made regularly.

Outside of this limited locality no isolations of the nematode have been accomplished, even though a diligent search has been made in numerous other parts of New Jersey and in Pennsylvania. In addition to the Japanese beetle, several other species of insects are susceptible to infection by *Neoaplectana glaseri*. We are informed by Glaser, McCoy, and Girth

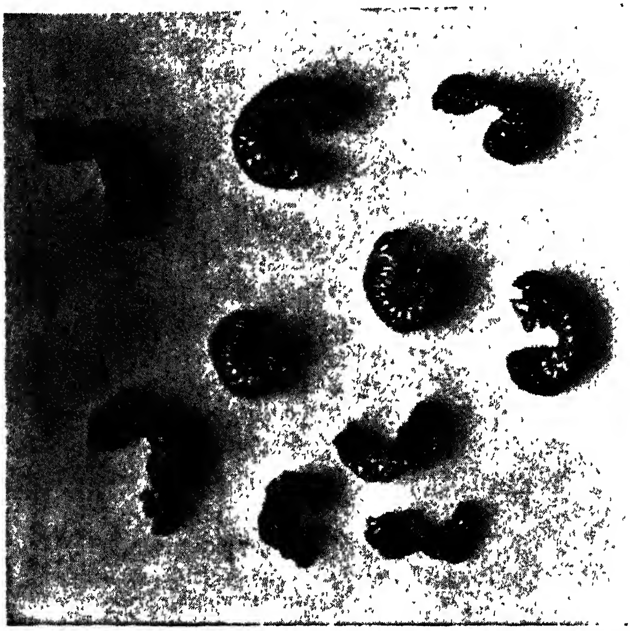


Fig. 202. Japanese-beetle grubs in various stages of parasitism by *Neoaplectana glaseri* Steiner. The shriveled cadavers signify that the interiors have been devoured. (*Courtesy of R. W. Glaser.*)

(1940) that parasitism of the larvae of *Anomala orientalis* Wth. occurs with a facility at least equal to that observed in the Japanese-beetle grubs. Other susceptible insects include the larvae of the following: *Autoserica castanea* (Arrow), *Macrodactylus subspinosus* (Fabr.), *Ochrosidia villosa* (Burm.), *Cotalpa* sp., *Cotinis nitida* (Linn.), *Xyloryctes satyrus* (Fabr.), several species of *Phyllophaga*; the larvae of the white-fringed beetle, *Pantomorus leucoloma* (Boh.); and the larva of the European corn borer, *Pyrausta nubilalis* (Hbn.). Some attempts to infect silkworms and armyworms have been unsuccessful.

Symptoms. Normal healthy grubs of the Japanese beetle are white in color, fairly active, firm to the touch, and have a good appetite at summer temperatures. When infected with *Neoaplectana glaseri*, the grubs have a diminished appetite, become flaccid, and are less active. They become colored and may be somewhat mottled in appearance; at first the coloration

is spotty, but later, just before and after death, it is more uniform. The most characteristic color is a rusty or ocherous brown; but dark-brown, light-brown, and dirty-white colorations also occur. Grubs that have turned black in color are usually not parasitized by nematodes.

To determine with certainty if a larva is parasitized by *Neoaplectana*, a microscopic examination is necessary. The body contents of a parasitized grub are usually swarming with nemas. As many as 2,400 infective-stage nematodes have been recovered from one Japanese-beetle grub—usually the number is about 1,500. Occasionally nemas of the genus *Diplogaster* are encountered. In dying and recently dead larvae and pupae, nearly all stages of *Neoaplectana* exist. In insects that have been dead for a long time, the dark second-stage nemas predominate.

Experimentally the average length of time from infection to death is approximately 11 days, and infection usually results in death. Occasionally a grub becomes parasitized very lightly—perhaps only one nematode originally becomes successfully established—but the host may eventually be killed.

Life History. The infective second-stage forms are acquired by the Japanese-beetle larva through the mouth. After entering the insect the nematodes soon develop into mature males and females and copulate. The female is ovoviviparous, the eggs hatching within the uterus. After remaining in the uterus a while, the young nematode larvae pass out through the vulva one at a time. The average female produces about 15 young, which are discharged into the alimentary tract of the host. Under optimum conditions each generation requires from 5 to 7 days for completion. As the new generation develops within the grub, the parasites may become so numerous that the insect dies and the nematodes invade the entire body. The worms usually pass through one or two more generations in the cadavers of the host, consuming nearly all the contents and leaving only a sac formed by the integument and the head capsule, and filled with a thin fluid swarming with larval parasites. Since further nematode development continues in the dead host, the nematode is considered a saprozoic or semiparasitic organism rather than a true parasite. Glaser, McCoy, and Girth (1940) have suggested that *N. glaseri* is a species in transition from the free-living saprozoic mode of existence to a parasitic one. As many as three generations may occur within one host, two of these appearing after the host's death—a behavior not characteristic of the nematodes of vertebrates.

In the older cadavers the second-stage nemas are the predominant type. These invade the soil and remain in a free-living state until they are ingested by another grub. Sometimes exceptionally large female nemas, which may produce enormous numbers of eggs, are found in the beetle larva.

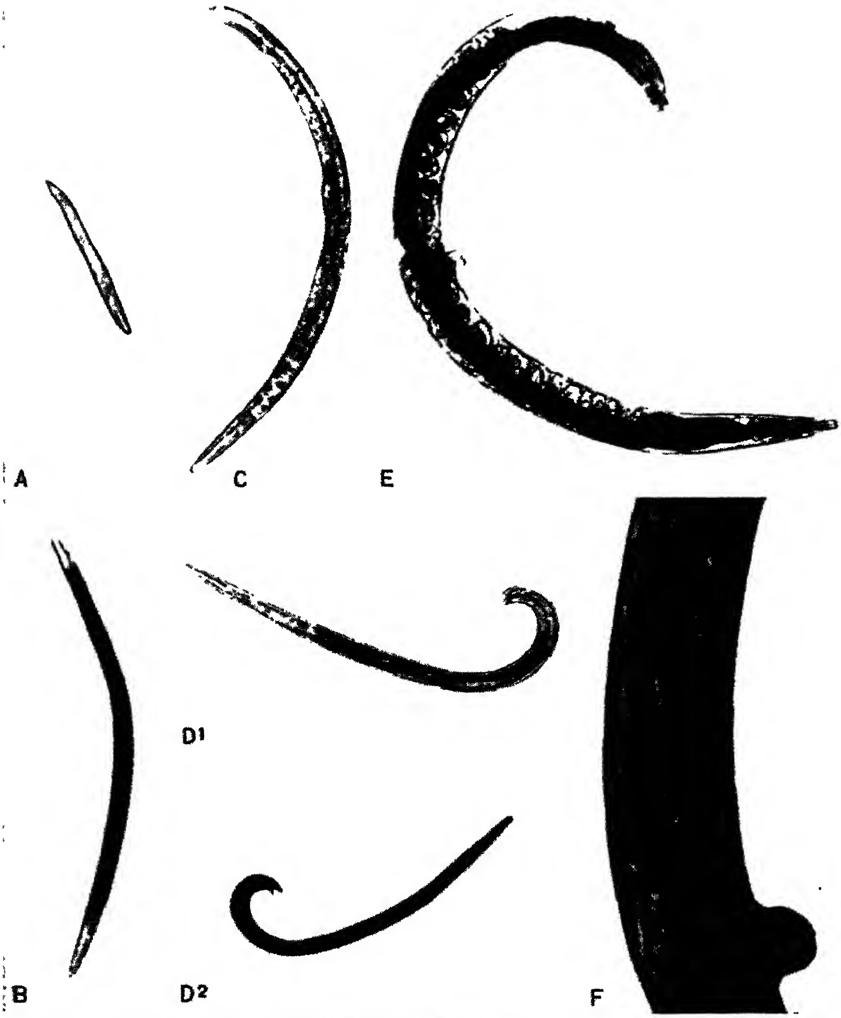


Fig. 203. Various stages of *Neoaplectana glaseri* Steiner. *A*. Newly hatched first-stage larva. This form develops into form shown in *B* after growth and molting. *B*. Second-stage larva, which escapes into the soil and is free-living. When mature, this form is also the invasive, or infective, stage, which penetrates through the oral cavity of normal beetle grub. Within the beetle grub the nematode again becomes parasitic, molts, and changes into the form shown in *C*. This preadult stage develops further, molts, and transforms into either the adult male form shown in *D* (two views, one taken by transmitted and one by reflected light), or into the adult female form shown in *E*. *F*. A section of an adult female has been enlarged, showing the ova and embryo within the uterus, and the vulva, through which the young larvae are expelled, protruding to one side. (*Photographs courtesy of R. W. Glaser.*)

As long as conditions are favorable and there is an abundant food supply in the insect, the life cycle of the nematode is completed in three molts, the third stage being omitted (Glaser, McCoy, and Girth, 1940). When the food is exhausted and conditions for continuous development are unfavorable, growth ceases at the end of the second stage, the alimentary tract empties, the body becomes more slender, and the parasite changes into the third-stage larva. In making this change, the cuticle of the second-stage larva is retained and the enclosed third-stage larva is said to be "ensheathed" or, as Fuchs (1915), Bovien (1932), and others call it, the "dauer" stage. In the case of *N. glaseri* the sheath is not very tenacious and is lost soon after the larva assumes a free-living existence in the soil, where it may persist, in a more or less active condition, for at least 2½ years.

According to Glaser (1932), the life history of *Neoaplectana glaseri* is essentially the same when the worm is reared on an artificial medium as it is in its insect host. Ensheathing can be brought about by placing the nematodes in an environment favorable for survival but not for growth to the preadult stage. This is done by removing the nematodes from the cultures, washing, and keeping them in an isotonic salt solution until they have ensheathed.

Cultivation. It was Montaigne who reminded us that "man cannot make a worm," but, after many fruitless attempts, Glaser (1931) did succeed in cultivating *Neoaplectana glaseri* on an artificial medium. The base of this medium was veal-infusion agar, having a reaction of pH 7.4. A day before the medium is to be inoculated with nematodes, 2 milliliters of a 10 per cent dextrose solution are added to a sterile Petri dish. The veal-infusion agar is poured into the dish and mixed with the sugar solution. When cool, the surface of the medium is flooded with a heavy suspension of a living pure culture of baker's yeast. The plate is incubated at room temperature until the yeast has grown uniformly over the entire surface of the medium. This takes about 24 hours, after which the nematodes—initially a washed suspension from infected grubs—are inoculated into the medium. The entire culture is then incubated at room temperature (24 to 27°C.).

The nematodes feed upon the living yeast cells until after about 2 weeks the yeast is depleted and the culture must be transferred to fresh medium. After about the seventh or eighth transfer on this medium the culture tends to die out, the nematodes failing to produce young. If the worms are passed through grubs several times they may regain their ability to survive seven or eight transfers on artificial media. If, however, supplementary food materials, such as desiccated cow's ovaries, are added to the medium it is possible to maintain the nematodes for many more transfers.

In 1940, Glaser described a test-tube method of growing the nematodes in yeast-free and bacteria-free cultures. Using aseptic techniques, mouse, beef, or rabbit tissue (ovary or kidney) was placed at the base of a veal-infusion-agar slant, and the evaporation from the tube was held to a

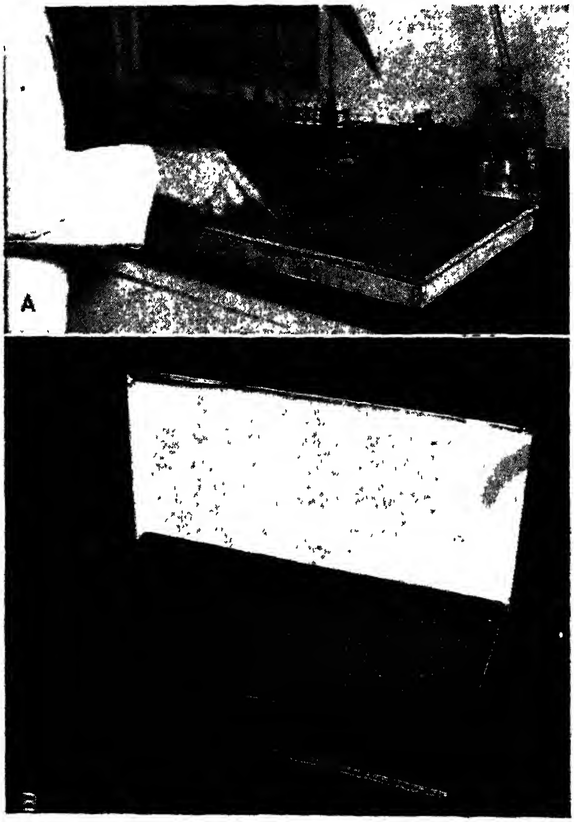


Fig. 204. Potato-mash culture. *A*. Preparation of potato mash and leveling it off preparatory to inoculation. *B*. Culture tray, with lid tilted, filled with potato mash, one of the media suitable for the rearing of *Neoaplectana glaseri* Steiner. (*Courtesy of R. W. Glaser.*)

minimum. In this, and in a similar liquid medium, the worms grew well, the tissue being almost entirely digested in from 18 to 24 days.

The two media just described, though excellent for maintaining stock cultures, are too expensive and do not yield large enough quantities of material desirable for field distribution. Accordingly, McCoy and Glaser (1936) developed a medium using fermented potato mash. This medium was placed in trays and inoculated with 200 to 400 thousand nematodes

from veal-infusion-agar plates. After an incubation period of from 6 to 10 days at about 21°C., a yield of about 4 million nematodes per tray was obtained. Used in a similar way but more productive and convenient was a medium developed subsequent to this by McCoy and Girth (1938), in which an infused veal pulp was used as a base.

The veal-pulp medium was kept from bacterial decomposition (bacteria are easily introduced with the nematodes) by the use of sodium methyl-*p*-hydroxybenzoate, with or without the addition of dilute formaldehyde solution, added as a preservative. Lean veal was used in the preparation of the medium, and this was ground through a food chopper, mixed with twice its weight of distilled water, and held in the refrigerator for 18 to 24 hours. The infusion was then poured through a flannel cloth and the pulp was mixed with preservative and placed in culture dishes. The nematodes were inoculated at a rate of about 600 nematodes per square centimeter of culture surface. After inoculation the cultures were incubated at a temperature of about 21°C. for approximately 7 days. The yield ranged from 9,000 to 12,000 nematodes per square centimeter of culture area.

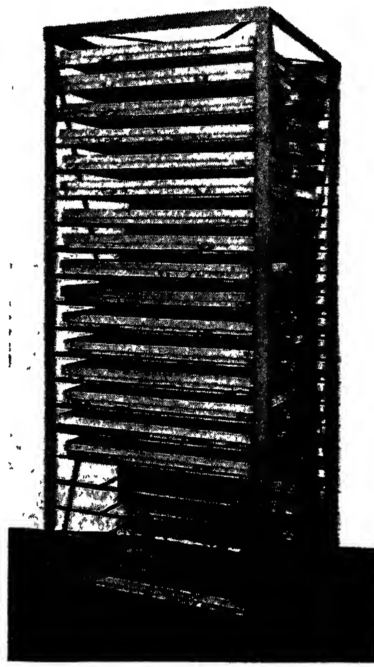


Fig. 205. Method of incubating and storing culture trays during the mass production of *Neoaplectana glaseri* Steiner. Two trays are placed on each shelf of the stall rack. (*Courtesy of R. W. Glaser.*)

Use as Control Measure. With the development of methods for propagating *Neoaplectana glaseri*, state (New Jersey) and Federal agencies about 1934 became interested in the possibility of using nematodes as a means of controlling the Japanese beetle in eastern United States. In cooperation with the Rockefeller Institute, these agencies conducted field experiments, the results of which indicated the potential value of nematodes as a control factor. In general, however, it appears that the widespread use of nematodes has had certain drawbacks that have made it impractical when compared with other means of control, including the use of the milky diseases of the Japanese beetle. In any case parasitization of the beetle by the worms, when it does occur, cannot but be beneficial.

In 1940 Girth, McCoy, and Glaser reported the results of 2½ years of field experiments conducted in New Jersey. Seventy-three tests were performed with ensheathed nematode larvae during this period. Two methods of treatment were used: a subsurface treatment in which a certain number of nematodes were placed in a small hole under the sod of the turf being treated; and a surface treatment in which the nematodes, in an aqueous suspension, were applied from a sprinkling can or spray tank onto the surface of the soil. More satisfactory results were obtained when the latter method was used.

The percentage of total population of Japanese beetles parasitized ranged from 0.3 to 81.5 per cent, depending on soil moisture, nematode dosage, soil temperature, and density of beetle population. The optimum conditions were (1) a soil temperature (at 1.5 inch depth) of 60°F. or higher; (2) soil moisture of 20 per cent or higher, with the soil not flooded; (3) a heavy host population; and (4) turf or other permanent cover. Nematode infestations have survived under field conditions for 8.5 years when the host population was maintained by stocking it with fresh grubs. Girth, McCoy, and Glaser found that the parasite was able to maintain itself under natural field conditions for 6.5 years, with a low host population existing for 5 years of this period. They also reported that nematode infestation in nature is spread by the flight of parasitized adult beetles and by the migration of nematodes and nematode-infected grubs through the soil.

The use of *Neoaplectana glaseri* as a control agent is one of the few instances in which nematodes have been used artificially in the control of insect pests. The role of nematodes generally in the natural destruction of noxious insects is known to be significant, and these worms probably supplement the activity of parasitic insects and predators. Such a conclusion as to the value of this type of control is certainly indicated by such reports as those from Japan, where mermithid infestations in leafhoppers have been reported very high (40 to 70 per cent) in certain years. Similar high infestations have been observed in certain scolytid grubs in Europe. Other examples have been cited by Oldham (1933).

Other Semiparasitic Nematodes and Novitious Parasites

Similar to *Neoaplectana glaseri* Steiner is *Neoaplectana chresima* Steiner, originally found near Moorestown, New Jersey, in dead and dying corn earworms, *Heliothis armigera* (Hbn.). It has also been found in larvae of the Japanese beetle, *Popillia japonica* Newm., and experimental infections have been produced in five additional insect species. The biology of this nematode has been described by Glaser, McCoy, and Girth (1942), who were able to cultivate it on artificial media. With certain exceptions,

its life cycle is similar to that of *N. glaseri*. In the Japanese beetle infection by *N. chresima* causes the insect to lose its appetite and become sluggish, and the grub cadavers assume a dirty dull yellow color. The larval contents become fairly viscid and have a dirty-yellow hue. Experimentally, Japanese-beetle grubs die about 4 days following exposure to soil or food contaminated with *N. chresima*. The possible use of this parasite in the control of its insect hosts has not been investigated.

Neoaplectana bibionis Bov. is a parasite of dipterous insects in Denmark. In the third stage of its development, dauer larvae are formed, which remain unchanged in the gut of the insect host until the latter eventually dies from other causes. When this happens, the nematode larvae move into the host's tissues, continuing their development until a large population is built up. When the insect cadaver is consumed, the young nematodes migrate out into the soil and become dauer larvae. *Neoaplectana affinis* Bov. also occurs in Denmark and parasitizes the same insects as does *N. bibionis* (Bovien, 1937).

Diplogaster labiata Cobb occurs in the elm borer, *Saperda tridentata* Oliv., and was originally found near Manhattan, Kansas. It reproduces in the intestine of the living adult insect and may accumulate in sufficient numbers to rupture the gut and kill the beetle. Infected female hosts are usually sterile (Merrill and Ford, 1916). Some species of *Diplogaster* may be associated externally with insects. Bovien (1937), for example, describes two species (*D. stercorarius* and *D. magnibucca*) that are carried by the dung beetle, *Aphodius fimetarius* (Linn.). The favorite site of attachment of these nemas is the lower side of the beetle's elytra at a point where the latter are attached to the body of the insect. Other species (*e.g.*, *D. aphodii*) may be found as endoparasites of dung beetles.

Pristionchus aerivora (Cobb) is best known in its relationship to termites, *e.g.*, *Leucotermes lucifugus* Rossi, but it has also been reported from dead pupae of the corn earworm, *Heliothis armigera* (Hbn.), and in dead pupae of the roseleaf beetle, *Nodonota puncticollis* (Say). In the case of the termite, the nematodes are found only in the head of the insect and vary from 1 to 75 per infected insect. When the infection is a heavy one, the termite becomes sluggish and dies, after which the nemas reproduce in the carcass. The specific name of this species is derived from the habit, shared by some other nematodes, of swallowing air which may be seen to pass down the esophagus to the anterior end of the intestine where it is absorbed (Cobb, 1915).

The genus *Rhabditis* contains a large number of saprozoic free-living species, species that live in terrestrial snails and worms, species that inhabit manure heaps or the galleries of bark beetles where they encyst on the exterior of the beetles, and a few species that live for a time in the

bodies of certain insects. An example of the latter is *Rhabditis janeti* (Lac. Duth.), which invades the salivary glands of the ant, *Formica rufa* Linn. The host-parasite relation here has not been clarified. Fuchs (1937) has separated out from the genus *Rhabditis* the subgenus *Parasitorhabditis*, the adults of which live in the galleries of bark beetles. For example, he distinguishes numerous varieties of *Parasitorhabditis obtusa* Fuchs that are associated with different species of these beetles both internally and externally.

Other species of nematodes having semiparasitic relations to insects have been reported, but the life histories of most of these are incompletely known.

NEMATODE PARASITES OF THE BODY CAVITY AND TISSUES OF INSECTS

This group of nematodes is considered by Christie (1941) to include the oldest parasitic nematodes in the sense that their progenitors were the first to assume a parasitic mode of life and have gradually become highly adapted to this way of living. Those species which parasitize the body cavity and tissues of insects are, for the most part, included in the families Tetradonematidae, Mermithidae, and Allantonematidae. A few selected examples from each of these families will be considered briefly in the pages that follow.

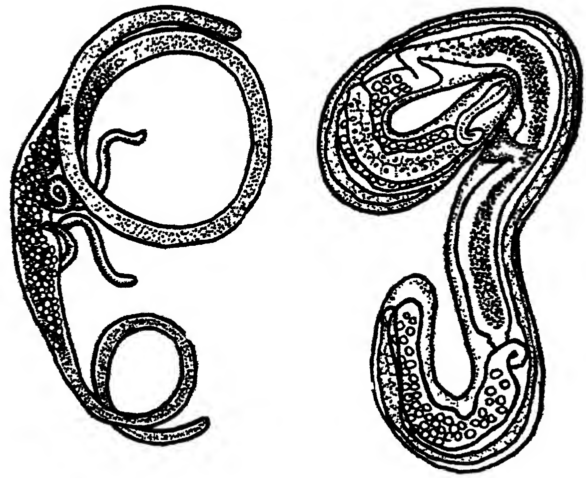
Tetradonematidae

Only two species of tetradonematids associated with insects are well known: *Tetradonema plicans* Cobb and *Aproctonema entomophagum* Keilin.

The first of these species, *T. plicans*, was found as a parasite of the larval, pupal, and adult stages of the gnat *Sciara coprophila* Lint. in Kansas (Hungerford, 1919). Each insect harbored an average of 10 nematodes. The adult stage of the worm is passed in the body of its host, where it also lays eggs. The exact route of infection is not known with certainty, but it has been assumed that the eggs are swallowed and hatch and that the nematode larvae penetrate through the wall of the gut into the body cavity. It is possible, however, that the larvae enter the host by penetrating the body wall. Once within the body cavity, the young nematodes develop rapidly and copulate, and the females lay eggs before the insect pupates. If the host larva is infected rather early in its life, the chances are that it will succumb before pupation occurs. In such individuals the fat tissue is consumed, and the body of the insect is more transparent than is normally the case. When there is a light infection the larval insect may pupate, and the pupa may die, or metamorphosis may continue until the adult emerges. The infected adults

are fairly normal in their activity and appearance, but they lack functional reproductive organs.

Aproctonema entomophagum Keilin was discovered in larvae of a gnat, *Sciara pullula* Winn., in England. According to Keilin and Robinson (1933), each infected host usually harbors several female nematodes and a varying number of smaller males. After the parasites reach maturity in the body cavity of the insect, they copulate. After this the males die



A *B*

Fig. 206. *A. Tetradonema plicans* Cobb, a parasite of the gnat *Sciara coprophila* Lint. An egg-laying female with males attached. (*Redrawn from Hungerford, 1919.*) *B. Aproctonema entomophagum* Keilin, a parasite of the gnat *Sciara pullula* Winn. A spermatized female. (*Redrawn from Keilin and Robinson, 1933.*)

and the females emerge by penetrating to the outside, whereupon egg laying begins almost immediately. When egg laying ceases, the female dies. After hatching, the young nematode larvae invade a new host, apparently by penetrating the body wall. Early infection of the larval insect usually portends its death; late infection may enable the insect (at least the female) to complete its metamorphosis which, however, may be delayed. The infected adult females do not have functional reproductive organs.

Mermithidae

The mermithids constitute a noteworthy group of many insect-parasitizing species. Most of them are very slender organisms, in most cases not longer than 10 to 20 centimeters in length, with the males usually shorter than the females. Their color is generally white because of the transparent cuticle and the presence of a large fat body; some species are faintly colored

with a yellowish, brownish, or blackish hue. The cuticle may be thick or thin. Those mermithids having thick cuticles parasitize terrestrial insects; those having thin cuticles inhabit the body of aquatic insects (Filipjev and Stekhoven, 1941).

A B

Fig. 207. The European earwig, *Forficula auricularia* Linn., parasitized by a nematode, *Mermis* sp. *A*. Part of coiled nematode, shown protruding from fore part of host's abdomen. *B*. Nematode emerging from earwig. (*From Crumb et al., 1941; courtesy of B. J. Landis, U.S. Department of Agriculture.*)

We shall not here attempt to generalize regarding the nature of the biological relationships between mermithids and their insect hosts, nor shall we try to give a general picture of the pathology resulting from the parasitization. Instead these points will be dealt with in the course of considering a few of the better known examples of the mermithid parasites.

Mermithid Infections in Grasshoppers. Grasshoppers are subject to parasitization by several species of mermithids. In the United States

two species of nematodes are prominent in this regard: *Agamermis decaudata* C., S., & Ch. and *Mermis subnigrescens* Cobb. Although *A. decaudata* is sometimes found in insects other than grasshoppers, *M. subnigrescens*

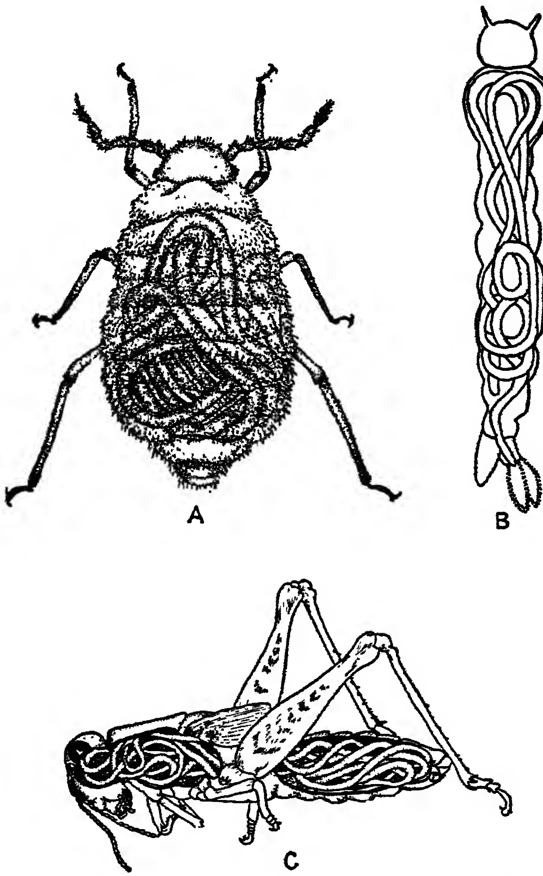


Fig. 208. Examples of insects parasitized by nematodes. *A*. A root aphid (*Anoecia*) infected with an undetermined nematode. (*Redrawn from Davis, 1916.*) *B*. Larva of *Aëdes aegypti* (Linn.) infected with two nematodes (*Mermis* sp.), one of which is about to emerge via the anus. (*Redrawn from Muspratt, 1945.*) *C*. Grasshopper nymph (*Melanoplus*) containing one fully grown female *Agamermis decaudata* C., S., & Ch. (*Redrawn from Christie, 1936.*)

appears to be strictly a grasshopper parasite. Both species occur in the northeastern and north central parts of the United States. Important papers on these nematodes have been published by Christie (1936, 1937) and by Cobb (1926, 1929), and the following account is based on these reports.

Agamermis decaudata has been found in grasshoppers (both Locustidae and Tettigoniidae), crickets (Gryllidae), and occasionally in leafhoppers and in beetles. So far as is now known, the species of grasshoppers most commonly serving as hosts are *Melanoplus femur-rubrum* (DeG.) and *Conocephalus brevipennis* (Scudder).

The free-living stages of *A. decaudata* inhabit small cavities in the soil usually from 5 to 15 centimeters below the surface. The adults in such cavities generally consist of one female and up to eight males coiled together so as to form a "knot." After copulation, egg laying takes place and, in Virginia, continues from about the first of July until cold weather sets in during the fall. Cleavage and embryonic development occur after the eggs are laid, and the first molt takes place within the eggshell; most of the eggs laid during a summer do not hatch until the following spring. The newly hatched second-stage larvae are at once infective as they migrate to the surface of the soil and climb grass and other low vegetation when it is wet with dew or rain. Newly hatched grasshopper nymphs are sought out and their body walls penetrated by the nematode, which thus gains entrance to the body cavity of the insect. The penetration usually takes place under the edges of the pronotum, between the abdominal segments, or at other locations where the integument is thin. Entrance through the chitinous covering is effected by the use of the nematode's stylet, "probably aided by the dissolving action of a chitin solvent secreted by one or more of the most anterior esophageal glands" (Christie). As the nema enters the insect, the posterior two-fifths of its body length—which up to this point has served as a propelling and food-storage organ—breaks off at a given spot (the "node") and is left outside. Within the body cavity of the grasshopper the parasite grows rapidly and undergoes pronounced external and internal morphological changes. The intestine, which serves as a reservoir for nutrient materials, assumes an enormous size, filling all the body-cavity space not occupied by the other organs. There is usually only one parasite per host. About 1 to 1½ months later the males force their way through the body wall of their insect hosts, followed 1 or 2 months later by the females. The worms fall to the surface of the ground and enter the soil where during the winter the males and the females remain separate, each individual forming a separate "knot."

The next spring the nematode undergoes its final molt, the males seek females, and copulation takes place. About the first of July egg laying begins and continues until interrupted by cold weather. The next spring the year-old female continues laying its eggs until, toward the close of the summer, its reserve food supply has become exhausted. Most of them do not survive a third winter in the soil.

The pathology of the infection by *Agamermis decaudata* is not very

apparent from external examination of the host grasshopper. The insects sometimes have distended abdomens and may appear sluggish and incapable of sustained flight. Internally, the gonads are visibly affected, particularly in the females. The ovaries are always greatly reduced in size, and infected female grasshoppers are probably not capable of laying eggs. The host grasshopper always succumbs when the parasite emerges from it.

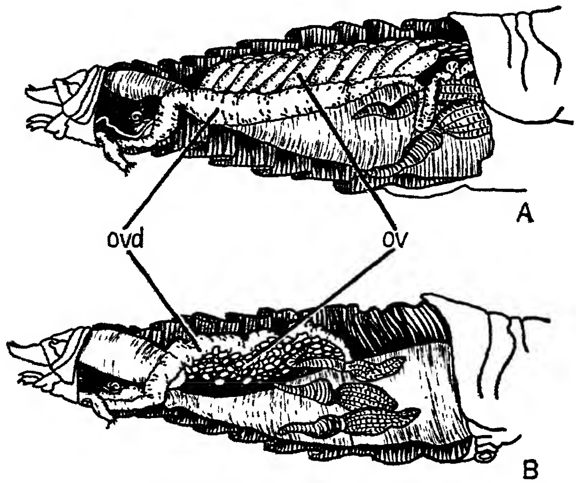


Fig. 209. Effect of mermithid nematodes on their hosts. Dissections of adult female grasshoppers, *Melanoplus femur-rubrum* (DeG.), showing effect of parasitization on reproductive organs. *A*. Normal grasshopper. *B*. Grasshopper parasitized by *Agamermis decaudata* C. S. & Ch. (*Redrawn from Christie, 1936.*)

Mermis subnigrescens Cobb has been found infecting nine different species of grasshoppers, including both Locustidae and Tettigoniidae, and two additional species have been experimentally infected. Attempts to infect other insects, such as crickets, have been unsuccessful.

The life cycle of this nematode is similar to that of *Agamermis decaudata* C., S. & Ch. but differs in one important respect. Instead of depositing its eggs in the soil, the gravid females of *Mermis subnigrescens* migrate to the surface of the soil, climb up the vegetation, and deposit their eggs thereon. The eggs are swallowed by grasshoppers which feed on the vegetation. Upon reaching the insect's alimentary tract, the eggs hatch, and the young larvae penetrate the wall of the gut and enter the body cavity where they continue their development. Since the grasshoppers become infected while feeding, they are vulnerable throughout their entire life. As the nymphs grow older, they eat more vegetation, and the chance that the insect will become infected is correspondingly greater.

A hundred or more parasites may be harbored by a single grasshopper; usually, however, a host contains from 1 to 5 parasites.

Within the body cavity of the host, the male nematodes remain from 4 to 6 weeks, and the females from 8 to 10 weeks. The parasites emerge by forcing their way through the body wall of the insect, after which they

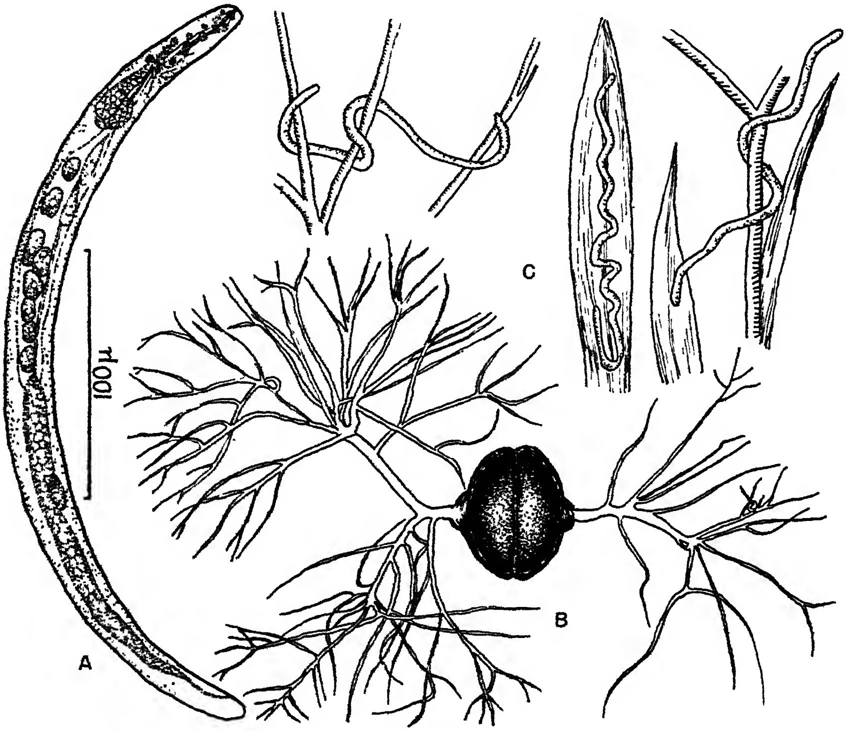


Fig. 210. *Mermis subnigrescens* Cobb, a parasite of grasshoppers. *A*. Recently hatched larva. *B*. Egg showing the branched byssus, which may vary in form. *C*. Females depositing eggs on grass in the field. Not according to scale. (*Redrawn from Christie, 1937.*)

enter the soil. The emergence of *M. subnigrescens* results in the death of the host. If the insect happens to harbor parasites of different ages, all those that are too immature to escape and survive in the soil perish with the host when the older nematodes emerge.

Unlike *Agamermis decaudata*, individual *M. subnigrescens* remain separated in the soil and rarely do a female and one or more males coil up into a knot. Copulation may take place, but it is not necessary and females reared in the absence of males produce viable eggs. According to Christie (1937), most of the nematodes emerge from the host during

the summer and autumn and molt the following April. By July the females begin to exhibit a brownish color due to accumulating eggs, and by September they are nearly black in appearance. Although the eggs are viable at this time, they are not laid until the following spring, usually beginning in May and continuing throughout the summer until the end of July or August. Egg laying takes place in the daylight during periods of rain or heavy dews when the vegetation is wet.

Before the parasite emerges, causing the death of the host, certain changes are brought about in the body of the grasshopper as a result of the parasitization. The development and function of the gonads, particularly the ovaries, is suppressed. This effect is more pronounced in those insects which harbor greater numbers of parasites. The growth of both male and female grasshoppers is considerably retarded, infected individuals remaining in the nymphal stages longer than uninfected insects.

Both *Agamermis decaudata* and *Mermis subnigrescens* are of economic importance in that they serve as factors in the natural control of the grasshoppers they infect. The hardiness of *M. subnigrescens* makes it particularly important as a means of control, since it is able to maintain itself in large numbers where the grasshopper population is consistently low. The latter species appears to be the more suitable of the two for colonizing, although very little investigation of such a possibility has been carried out.

Mermithid Parasites of Ants. For many years it has been known that numerous species of ants are often infected with mermithids which bring about more or less typical changes in their hosts. Sometimes the parasitized ant appears only slightly different from a normal individual—perhaps there is only a slight difference in color or the gaster is somewhat distended. On the other hand, the pathology resulting from the parasitism may be marked, modifying the external anatomy so that the infected individual is not identical to any normal caste but shows female, worker, or soldier characters in varying degrees only. Such ants are known as "intercastes."

Various types of intercastes occur, and these are sometimes designated by different names. "Mermithogynes" may be found in the genus *Lasius* where infected females resemble normal females but usually have a smaller head, shorter wings, and a partly distended gaster. Several different intercastes may occur in the genus *Pheidole.* These have been termed "mermithergates" and have a mixture of female, worker, and soldier characters. In most cases they resemble workers and soldiers. Five of these types have been recognized (Wheeler, 1928). Two types of intercastes have been found in *Pheidole pallidula* (Nyl.). One of these shows very little variation from normal; the other is a modified soldier type which

Vandel (1930) designates as "mermithostratiotes," reserving the term "mermithergates" for modified worker types.

The life cycles of the nematodes (*Agamermis, Hexamermis, Allomermis*, and others) that parasitize ants have not been studied to any great extent. The route of infection appears to be oral in some cases but not in others; in most instances it is unknown. Typical in this regard is the uncertainty in the case of *Allomermis myrmecophila* (Baylis), which parasitizes ants (*Lasius alienus* (Förster), *L. flavus* (Fabr.), and *I. niger*

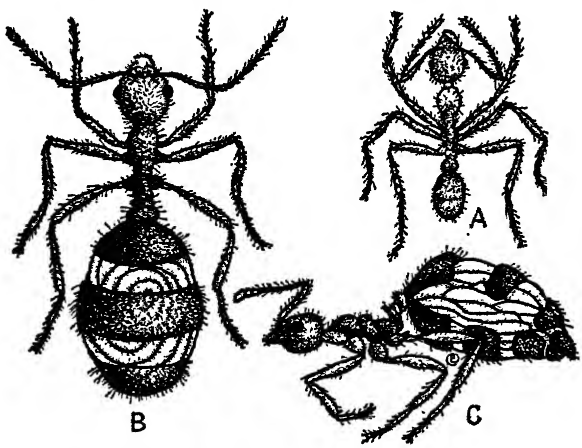


Fig. 211. Examples of mermithergate ants (*Pheidole*). *A*. Normal worker. *B* and *C*. Dorsal and lateral views of infected workers showing worm coiled in the abdomen. (*Redrawn from Wheeler, 1910.*)

(Linn.)) in England, Germany, and probably elsewhere. Crawley and Baylis (1921) believe that the ants become infected while in the larval stage, whereas Gösswald (1929, 1930) presents evidence that indicates that the eggs of the nematode are ingested. Similar uncertainty concerns the rest of the life history of this mermithid. It does appear that after the parasite has completed its development, it emerges from the insect, either through the anus or sometimes between two of the ventral plates of the gaster. It enters the soil and lays large numbers of eggs. Males have not been seen, and the females apparently are able to produce viable eggs without copulation.

Allantonematidae

The allantonematids constitute a large group of nematodes parasitic in insects and having a life cycle distinctly different from that found in any other group of nematodes. Judged from an over-all viewpoint, they constitute an important factor in the natural control of insects since not only do they kill large numbers of insects, but many of them sterilize

their hosts, thus preventing the laying of viable eggs. Consequently their aggregate destructive effect on insect populations must be considerable.

As Christie (1941) has described them, the adult gravid females occupy the body cavity of the insect, frequently in small numbers, often one per host. The eggs are either deposited here in the body cavity of the host or they hatch before deposition. In either case, the young larvae commence their development in the host insect, molting once or twice, depending on the species, and then escape from the host. This is accomplished either by entering the alimentary tract and passing out through the anus or by entering the female reproductive system and passing out through the genital aperture. In most cases, both male and female insects are infected by the nematodes. The free-living stage, usually of short duration, is passed wherever the host insect undergoes its early development. During this period, the worms molt at least once, in most cases probably twice, and become adults.

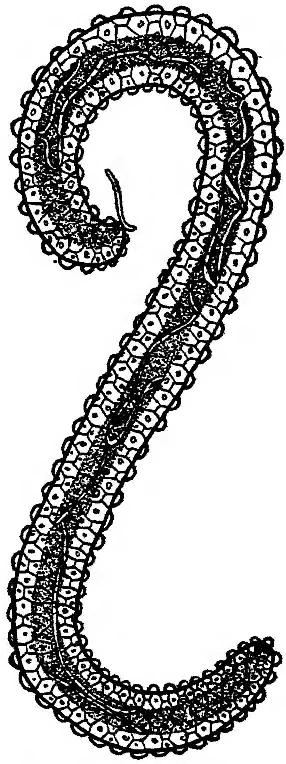


Fig. 212. The female reproductive system of *Sphaerularia bombi* Duf. The nematode's small body proper remains attached at one end of the prolapsed uterus. (*Redrawn after Leuckart, 1887.*)

Upon entering a new host, the female nematode increases greatly in size. When fully grown, the female is usually curved ventrally and assumes a sausagelike shape; there are, however, exceptions to this. If the female does not actually lay her eggs in the body cavity of the host, her uterus becomes distended with developing eggs and larvae, which fill a large part of her body, and some of her organs degenerate. Usually the larvae pass out through the vulva of the female into the body cavity of the insect. In some nematode species the size of the female does not increase enough to provide space for the expanding reproductive organs. In *Sphaerularia bombi* Duf., for example, the uterus is everted through the vulva, and the entire reproductive system develops outside the body of the nematode. This prolapsed uterus increases so much in size that the body proper amounts to little more than an attached vestigial structure, apparently without function.

As Christie points out in his discussion of the group, there are several deviations from this typical life cycle. In some cases, the males as well

as the females enter the body cavity of the insect. Sometimes neither the males nor the females become parasitic, this role being taken over by the larval stages. A few species have heterogeneous life cycles, having parthenogenic and gamogenetic generations.

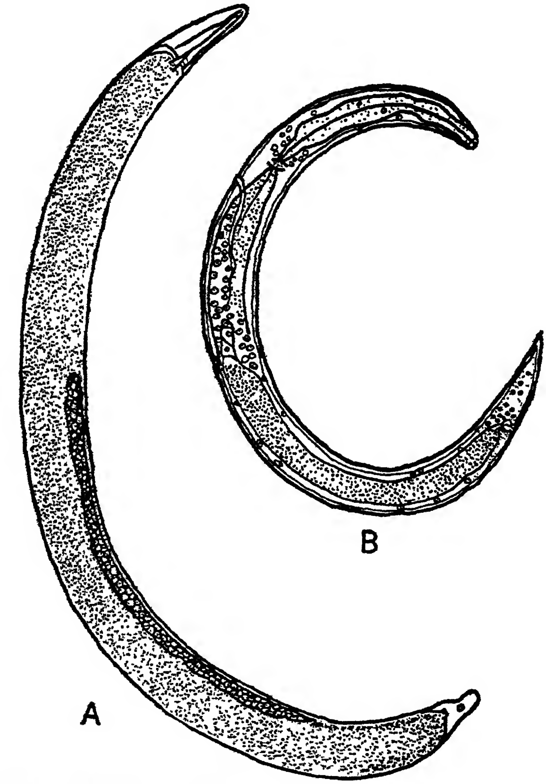


Fig. 213. *A. Chondronema passali* (Leidy), a parasite of the beetle *Popilius interruptus* (Linn.). Old larva from body cavity of host. (*Redrawn from Christie and Chitwood, 1931.*) *B. Allantonema mirable* Leuck., a parasite of the pine weevil, *Hylobius abiëtis* (Linn.). An adult preparasitic female after copulation. (*After Wülker, 1923.*)

It is difficult to select representative examples from this large group of parasitic nematodes. The best we can do, and keep within the limits of this chapter, is to mention the high points in the life histories of some of them and to point out some of the interesting biological and pathological relationships existing between them and their insect hosts.

Allantonematids Parasitizing Beetles. About 100 years ago Leidy discovered the nematode now known as *Chondronema passali* (Leidy) in the body cavity of the beetle *Popilius interruptus* (Linn.). A large

percentage of any particular beetle population may be infected, and each beetle may harbor from 500 to 1,000 parasites, which may be in all stages of development except adults. Once outside their hosts, neither the males nor the females become parasitic but remain in the galleries formed by the beetle in decaying logs and stumps. The eggs hatch in the body of the female worm, and the very young larvae enter the host possibly perorally.

The body cavity of the pine weevil, *Hylobius abiëtis* (Linn.), is sometimes found parasitized by *Allantonema mirable* Leuck. in Europe. Unlike most allantonematids, the fully grown female of this nematode is oval in shape, about 1.5 to 2 millimeters long and about 0.75 to 1 millimeter wide. It consists essentially of a sac filled with the uterus containing eggs and larvae. When the eggs hatch they pass into the body cavity of the host, undergo two molts, and eventually leave by penetrating the insect's alimentary tract and passing out through the anus. This escape is made in the vicinity of the place where the adult beetles lay their eggs so that the female nematode may later, after copulation, penetrate into the body cavity of the weevil larvae.

In 1921, Cobb reported finding a nematode parasite, which he named *Howardula benigna* Cobb, in the cucumber beetle, *Diabrotica vittata* (Fab.). It occurs less commonly in *D. trivittata* (Mann) and in *D. duodecimpunctata* (Fab.). The nematode larvae pass out with the eggs of the insect. Male beetles, as well as females, are infected, but the fate of the nematode larvae within the male is not known; it is possible that they are transferred to the female beetle during copulation.

The bark beetle *Ips typographus* (Linn.) is parasitized by at least two species of allantonematids: *Aphelenchulus diplogaster* (Linst.), and *Parasitylenchus dispar* subsp. *typographi* Fuchs. The bark beetle *Pityogenes bidentatus* (Hbst.) has been found to harbor *Aphelenchulus tomici* Bov. which, like *A. diplogaster* (Lint.), passes out of its host through the anus and undergoes its free-living development in the frass of the beetle galleries. Several species of the nematode genus *Bradynema* also occur in beetles.

Allantonematids Parasitizing Flies. One of the best known allantonematids parasitizing flies is *Tylenchinema oscinellae* Goodey observed by Goodey (1930) as a body-cavity parasite of the frit fly, *Oscinella frit* (Linn.), in England. The fly has three generations a year and the life cycle of the nematode is, accordingly, closely correlated with that of the insect—it also undergoes three generations a year. Within the body cavity of the insect the female nematode gives birth to living young. These young larvae accumulate in the hemocoele and continue their development until eventually they effect their escape from the host by penetrating the food reservoir of the fly's digestive system through the anus. After a

short free-living existence, the impregnated female enters the body cavity of the frit-fly larva, probably by the direct penetration of the body wall. The pathological effects of the parasitization are not apparent on the external characters of the host. Internally, however, both the male and female gonads are sterilized. Occasionally the fly gets the upper hand and is able to build up its gonads in a normal way; in such cases the nematode fails to grow and becomes degenerate.

Fig. 214. *Scatonema wülkeri* Bov., a parasite of *Scatopse fuscipes* Meig. *A*. An adult parasitic female a few days after entering host. *B*. Fully grown gravid female. (*After Bovien, 1932.*)

The small dipterous insect *Scatopse fuscipes* Meig. is known to be parasitized by the body-cavity parasite *Scatonema wülkeri*, described by Bovien in 1932. This nematode is of particular interest because its progeny can reach full maturity and even copulate inside the maternal uterus, or within the body cavity of the host. The life cycle of the parasite may be completed in a period of a few weeks while the host is still in the larval stage. The free-living stage is of short duration. The females penetrate the body wall of the fly larva at almost any point on the surface of the insect's body. Most of the host larvae contain but one parasite, some two, and a few have been found harboring three nemas. The parasitization does not appear to have any marked effect upon the adult host, and its presence does not result in the sterility of the insect. In the case of the host larvae, however, the parasites fill a good share of the body cavity, the fat body of the insect is consumed, and finally the insect succumbs to the attack of the parasites.

Heterotylenchus aberrans Bov. is a parasite of the body cavity of the onion maggot, *Hylemya antiqua* (Meig.), in Denmark from where it was reported by Bovien (1937). Each parasitized fly usually contains from one to four large adult females (gamogenetic generation) together with a larger number of small adult females (parthenogenetic generation). The gamogenetic females deposit their eggs in the body cavity of the host, where they hatch and where the larvae develop into parthenogenetic females. These females deposit eggs in the body cavity of the insect,

and from these hatch larvae of both sexes. When these larvae are ready to undergo their final molt, they penetrate the insect's ovaries and migrate to and accumulate in the oviducts, from which they escape through the genital aperture. The infected female flies are rendered sterile because their ovaries fail to develop; the male flies, although infected, probably retain their fertility. The fat tissues of the body are also greatly reduced. How the nematodes escape from the male insects, if they do, has not been determined. In their free-living existence, the nematodes reach maturity and copulate, after which the males die and the females enter the insect, presumably by penetrating the body wall.

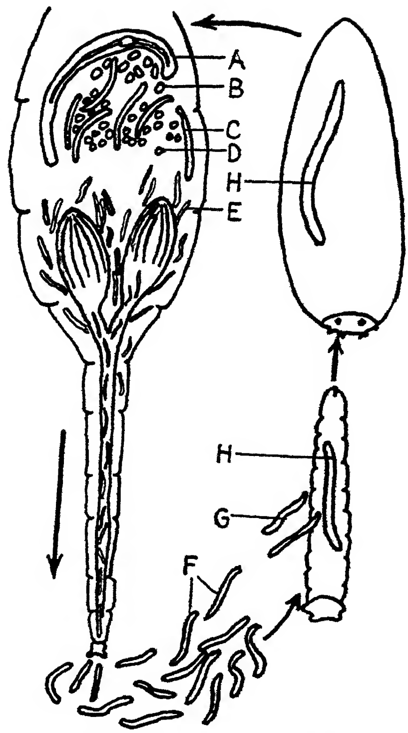


Fig. 215. A diagrammatic representation of the life cycle of *Heterotylenchus aberrans* Bov., a parasite of the onion fly, *Hylemya antiqua* (Meig.). The adult parasitic female of the gamogenetic generation (*A*) lays eggs (*B*), which develop into females of the parthenogenetic generation (*C*). These females lay eggs (*D*), and the resulting larvae (*E*) enter the reproductive organs of the female fly and pass out through the genital aperture. Outside the host these larvae develop into adults of the gamogenetic generation (*F*) and copulate, whereupon males die and impregnated females (*G*) enter fly larvae. While the fly matures and pupates, the female grows (*H*) to reach full stature (*A*) and lays eggs (*B*). (*Redrawn from Christie, 1941, after Bovien, 1937. Legend from Christie.*)

Another species of nematode parasitic in a dipterous insect is *Tripius gibbosus* (Leuck.) found in *Cecidomyia pini* (DeG.) in Germany. During the parasitic development of the female, the uterus is gradually everted through the vulva. The uterus continues to develop on the outside of the nematode until it forms an oval structure attached to the smaller body proper.

Fergusobia curriei (Cur.) occurs in gall flies of the genus *Fergusonina* in Australia. This nematode is also found in the galls produced by the insect and may be considered a true plant parasite. Some authorities regard its relationship to the insect as one of symbiosis (mutualism) rather than parasitism. This appears to be a very unusual case of combined parasitism and mutualism, depending upon whether the worm is in the plant or in the insect host.

Allantonematids Parasitizing Other Insects. One of the most interesting and remarkable of nematode parasites of insects is *Sphaerularia bombi* Duf., which parasitizes several species of *Bombus* (bumblebees), as well as *Vespa rufa* Linn. and *Vespa vulgaris* Linn., in Europe and in North America. Each host harbors one, or only a few nemas. *S. bombi* has a fairly typical allantonematid life cycle: Eggs are deposited in the body cavity of the host, and the larvae, after a period of development, pass out of the insect by way of the anus and enter the soil. Here they reach maturity, and copulate; the males die, and the females enter their new hosts, which are usually queen bees. Only the queen bees are parasitized because they hibernate in the soil and the nematodes gain access to them while the insects are penetrating the ground in the autumn. The ovaries of the infected queens are retarded in development and produce few or no eggs, and such queens probably never establish colonies.

After entering its insect host, the female of *S. bombi* undergoes little or no increase in size, but the uterus gradually is everted through the vulva. The everted uterus carries with it the other reproductive organs and the modified intestine or "fat body," and completes its development on the outside of the body proper. The prolapsed uterus increases to an enormous size while the original body of the nematode remains a small functionless structure that may become detached (see Fig. 212). The pathological changes in *Sphaerularia*-infected bees, particularly in the ovaries, have been described by Palm (1948), who considers the parasite to be a genuinely pathogenic worm.

The thrips *Aptinothrips rufus* (Gmelin) is subject to attack by *"Tylenchus" aptini* Sharga. This nematode deposits its eggs in the body cavity of the host, and the larvae eventually leave by way of the alimentary tract and anus. Other insects serving as primary hosts to allantonematid nematodes include aphids and earwigs.

NEMATOMORPHA

The Nematomorpha (or Gordiaceae) are in many ways similar to the Nematoda except that they are usually larger, more uniformly cylindrical, have bluntly rounded ends, and have a faintly colored cuticle. There are other structural and physiological differences, but in many respects their life cycle is similar to that of mermithids. Because of their superficial resemblance to thick hairs they are sometimes known as "hairworms." Insects, diplopods, molluscs, and crustaceans constitute their favorite hosts.

Gordiid larvae gain entrance into their insect hosts by being ingested along with the insect's food or water, and through ingestion by the insect of the encysted worm. After being ingested by the insect, the cyst bursts

and the larva escapes, perforates the intestinal wall of the host, and reaches the body cavity. If the host is large enough, the larva may accomplish its development in this situation. If, however, the insect is a small one, it may serve only as an intermediate host, and the worm will become an adult after the intermediate host has been ingested by the final host. After leaving their host, the male and female worms copulate and oviposition soon follows. The adult worms may live for 6 months or more in fresh water or in mud.

Those insects which serve as intermediate hosts of Nematomorpha are frequently small aquatic insects (*e.g.*, Chironomidae, Ephemeridae, Trichoptera). If these insects are not swallowed by another insect, the worms may remain in them for a year or more until the parasites finally disintegrate and become absorbed by the host's tissues. On the other hand, when the intermediate hosts are ingested by a carnivorous or by an omnivorous insect, the worms complete their development in the latter, or final, hosts. In the case of some of the worms that complete their development in terrestrial insects, the intermediate hosts may be tadpoles, young frogs that have just metamorphosed, and also snails. Filipjev and Stekhoven (1941) list the following groups of insects as hosts of Nematomorpha: Coleoptera (Silphidae, Tenebrionidae, Dytiscidae), Orthoptera (Tettigoniidae, Locustidae, Mantidae, Blattidae), Odonata, and Trichoptera.

The effects of Nematomorpha on their hosts are usually not great. Sometimes the fat body of the insect is decreased, and there may be other minor anatomical or histological alterations. Some insects (*e.g.*, Carabidae) may have their fat body reduced as a result of the parasitization, which may, in addition, lead to a degeneration of the reproductive system and parasitic castration of the host. The intestinal tract may also be arrested in its function. Some insects die when the worms escape from them; others may survive this process and continue their normal existence.

The Nematomorpha are widely distributed and are found in both marine and fresh-water environments, and, as far as insects are concerned, in both terrestrial and water-inhabiting species. Most of the forms in which we are interested here belong to the two families Gordiidae and Chordodidae. The single important genus of the first family is *Gordius*; the latter family includes the genus *Chordodes* and 7 or 8 other genera.

PLATYHELMINTHES

A few species of flatworms are known to be associated with insects, but in most of these cases the insect serves merely as an intermediate host. Examples of such worms and their intermediate hosts include *Dipylidium caninum* (Linn.), the dog tapeworm (in fleas), *Hymenolepis nana* (v.

Sieb.) (in fleas and probably certain beetles), *H. diminuta* (Rud.) (in certain lepidopterans, earwigs, fleas, beetles, and cockroaches), *Raillietina echinobothrida* (Meg.) (in ants), *R. cesticillus* (Molin) (in certain beetles), *Moniezia benedeni* (Mon.) (in certain mites), and *Choanotaenia infundibulum* (Bloch) (in the housefly and dung beetles).

An interesting case of flatworm parasitization of an insect has been reported by Subramaniam and Naidu (1944) in India. These workers observed a minute plerocercoid (*Sparaganum*) in the fat tissue of a sandfly (Psychodidae). The worm showed branching and segmentation and had a small club-shaped scolex. The nature of the relationship between the worm and its host has not been elucidated.

One of the few cases of an association between trematodes and insects is that in which *Prosthogonimus pellucidus* (v. Linst.) of fowl and ducks is found as cercaria in nymphs of dragonflies, especially *Libellula quadrimaculata* Linn. The birds become infected when they ingest the dragonflies.

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CHAPTER 14

APPLIED INSECT PATHOLOGY AND BIOLOGICAL CONTROL

Throughout this book reference has been made to the use of microorganisms in the biological control of certain insect pests. At no point, however, has there been an opportunity to discuss certain of the more general aspects of the subject; hence we shall attempt to do so in the present chapter. It is suggested that the reader consult again the section in Chap. 6 dealing with the epizootiology of insect diseases, since the material included there has a direct bearing upon much of that with which we shall be concerned here.

In approaching the matter of insect control through the agency of pathogenic microorganisms, it should be remembered that we are dealing with but a single part of the biological complex that concerns itself with the ecology of insect life. Furthermore the control of insects through the use of microorganisms frequently complements the type of control brought about by parasitic insects and by predators. Indeed many of the principles and laws governing the control of insect pests by means of other insects apply almost equally well to the control of insect pests by the use of microorganisms. Applied insect pathology (*i.e.*, the microbial control of insects) must therefore be considered as an integral part of that general field of entomological endeavor usually spoken of as "biological control."[1] Because of this, the entomologist will be likely to hold more or less the same attitude toward applied insect pathology that he will toward the broader subject of biological control generally.

Unfortunately the field of biological control as such is not fully recognized or adequately appreciated by the average entomologist. In some parts of the world, such as in Australia and in certain parts of the United States, extensive applications have been made of the biological method of controlling insect pests; even in these areas, however, the value of the field to agriculture is not generally realized. In relation to the

[1] In this chapter, as throughout the entire book, the word "control" is used as defined by Smith (1939), to indicate the maintenance of a population density below the point where economic injury to man's interests occurs. We should add that we consider the phrase "biological control" as a broad term including both natural control and control instituted by man. The term "microbial control" is convenient to use in referring to that part of biological control concerned only with microorganisms, differentiating it from that part concerned with insect parasites and predators.

amount of time, research, and money spent on other phases of economic entomology, the field of biological control has been rather seriously neglected. As it concerns the field of insect pathology itself, there has developed, in some uninformed quarters, a distinct reluctance to, and an antipathy for, the idea of using microorganisms to control insect pests. Such skepticism is, in part, understandable when it is remembered that many of the highly advertised attempts of the past resulted in discouraging failure. To be sure, many of these failures were the results of superficial and ill-advised tests, but since failure is frequently remembered longer and more vividly than modest success, the practical applications of insect pathology have, in the past, suffered some rather painful body blows. The disappointment in a few hastily tried attempts to control certain insects with certain microorganisms has blinded many to the potentialities that this method has when based on a firm scientific foundation and a thorough knowledge of the numerous factors involved. Initial reports concerning the efficacy of such forms of biological control were frequently overenthusiastic, and, after a few disappointing failures, the early optimism was replaced by undue pessimism. This gave rise to unjustified criticism regarding all phases of insect control through the use of microorganisms and a general lack of interest in the field which is only now being overcome.

Unquestionably the greatest stumbling block to the widespread use of microorganisms in the artificial control of insects is man's colossal ignorance of the subject and his inability to control the various environmental factors that play the dominant role in the ecology of the diseases concerned. It should be repeatedly emphasized that the microbial control of certain insects is being continuously maintained in nature without the help or the interference of man. In other cases, natural epizootics of disease break out from time to time and substantially reduce dangerous populations of insects. Therefore, whether the economic entomologist wishes to recognize it or not, microbial control of insects is effective, does take place *in nature*, and is of great over-all economic importance. The artificial use of microorganisms, on the other hand, has been a different matter, generally speaking. With few exceptions, man has succeeded with the microbial method only when the problem has been met forthright with sound scientific experimentation by competent but cautious investigators.

Comparison of Control by Entomophagous Insects and Entomogenous Microorganisms. It is quite natural that the first phase of the biological control of insect pests to have been adequately studied and developed was that concerned with insect parasites and predators. The latter, after all, came within the entomologist's own field and could be studied by methods and techniques already familiar to him. Microorganisms

were somewhat foreign to his specialty, and the techniques necessary to study them were greatly different from those to which he was accustomed. Understandably, therefore, the entomologist interested in biological control concentrated on the parasitic and predatory insects. Accordingly, it is wise for the insect pathologist to consider and to reflect from time to time on the knowledge gained as a result of these studies. This is particularly true with regard to what has been learned concerning insect populations. The subject of insect populations in relation to biological control has been well discussed by H. S. Smith (1939) as it pertains to insect parasites and predators. It is expedient for us, therefore, to consider here briefly the relations existing between populations of insects and microbial control, basing our discussion on Smith's remarks concerning biological control generally.

Smith likens the paradoxical stability and incessant change of animal populations to change in the weather, which varies from day to day but which over a longer period of time is very stable. These static and dynamic qualities of population densities must be carefully distinguished in considering the effectiveness of biological or any other control agencies, because the same type of environmental factor may affect one characteristic of populations differently from the way it affects another. Although the nature of the various agencies constituting biological control can never be independent of such physical factors as climate, according to Smith, we are nevertheless justified in considering the relation between population densities and these biotic agencies independently of the rest of the environment, since climate influences primarily the degree of their effect rather than the kind of effect.

The principal biological control agencies influencing the population densities of insects are insect parasites, predators, and microbial disease. The three main characteristics of these agencies appear to be potential reproductive capacity (biotic potential), the "effective" rate of reproduction, and the ability or likelihood of the agency to discover a susceptible host. As concerns insect parasites and predators specifically, this last characteristic has been stated simply as the power of discovery of the entomophagous insect. Although these three characteristics are commonly used in discussions pertaining only to insect parasites and predators, to a large degree they, as well as others, may similarly be used in discussing the effect of pathogenic microorganisms upon insect populations. In the latter case we approach what is essentially the field of epizootiology.

Smith has explained that since in a stable association only one parasite will mature per parent, the *potential* reproductive capacity of an insect parasite or predator, by itself, is of little or no importance in its relation to the population density of its host; that the *effective* rate of reproduction

of an insect parasite or predator is important in its relation to the intensity of change in oscillations and outbreaks; and that "in the steady state, since the mean rate of change of the host population is zero, the mean rate of change of the parasite's population also must be zero, and that this is true regardless of the population level. The per cent of hosts destroyed in the steady state by a parasite does not therefore give any indication of its effect on the average level of the host population."

Now in the case of microorganisms the parent-progeny relationship referred to in the preceding paragraph is not so apparent, and the statement that only one parasite will mature per parent must be interpreted somewhat differently. The fact remains that in a stable association in nature there will probably be a rather constant number of "progeny" for that particular species of pathogen. The similarity here between insect parasites and microbial parasites becomes less distinct, however, when it is remembered that microorganisms do not always need a host for survival or even for reproduction and may, under the right conditions, propagate themselves saprophytically outside their hosts for almost indefinite periods of time. Even those microorganisms which are obligate entomogenous parasites may, through the agencies of spores, accumulate in nature and build up to a considerable number. This, however, may properly be considered as a part of the *effective* rate of increase which, as Smith points out, cannot be disregarded since such influence as an entomophagous insect has on the rate of change of the population density of its host is determined by the difference between its effective rate of increase and that of its host. The same may be said of the pathogenic microorganisms.

In the case of entomophagous insects, the most important property in relation to its effect on the population density of its host is its searching ability, *i.e.*, its capacity to find or discover hosts. This searching ability of entomophagous insects has its counterpart in entomogenous microorganisms in the various mechanisms by which the latter are able to gain access to a susceptible host. This ability may be with the microorganism itself or with its insect host. For example, the spores of many entomophthoraceous fungi possess adherent qualities that enable them to adhere to the cuticulum of their hosts to a greater or lesser degree. The insect host, on the other hand, may alter the invasive ability of the microorganism by the nature or the peculiarity of the portal of entry it affords the pathogen, or by the presence or absence of a specific immunity against the pathogen. The entire point may be signified as pertaining to the means that the microorganism has of distributing itself. Of course, the type of host dispersion has an important bearing on this situation. In fact, as with entomophagous insects, a microorganism's efficiency as a

biological control factor depends largely upon a combination of two qualities: host dispersion, and the microorganism's power of distributing itself or being distributed by agencies such as wind and water, or through contact. Applying it to microorganisms we are, moreover, able to concur with Smith's general conclusion in this connection, that, other things being equal, host insects having the colonial type of distribution can be controlled by entomophagous insects (and entomogenous microorganisms) which rank relatively low in their power of host discovery, whereas hosts whose population tends toward uniform scattering can be controlled only by factors having a high power of host discovery. The effectiveness by which we can facilitate a microorganism's distribution, therefore, may be of extreme importance in determining its effectiveness as a biological control agent.

One may be inclined at this point to compare the distribution powers of microorganisms with the low power of host discovery of insect predators which, in general, cannot be considered to offer as much promise as a biological control agency as do insect parasites. However, unlike microorganisms, insect predatory larvae have low powers of locomotion, and they must find a succession of hosts in order to reach maturity. Microorganisms, on the other hand, may be rapidly and widely distributed by such physical agencies as wind and fomites, and in general need to find only one host in order to complete their development and reproduce themselves.

Disease as a Factor in Insect Ecology. From the foregoing paragraphs it is clear that the biological control of insect pests is fundamentally a problem of populations. Smith (1935) further points out that this problem "is essentially ecological in nature because it has as its aim the modification of the biotic characteristics of the environment of the species in such a way as to influence its population density." Insect ecologists generally recognize such physical agencies as light, temperature, and humidity (to be considered later in this chapter), and such biotic agencies as predators and parasites as important ecological factors. Most insect ecologists, however, have either ignored the ecological significance of disease or have given it a mere passing reference. Although throughout the pages of this volume we have in a general way stressed the importance of disease as a factor in insect ecology, it is our purpose here to call special attention to this aspect of the subject and to highlight what we feel is one of the most important truths concerning the relation of insect pathology and insect ecology, namely, that a knowledge of insect diseases (whether or not it has to do with control) is of fundamental and far-reaching importance in the study of insect ecology. To be sure, disease among insects is closely related to, and dependent on, the dynamics of such abiotic factors as

temperature and humidity, but in so far as is possible, it is nevertheless probably as worthy of distinct consideration as are such biotic factors as insect parasites and predators.

In the preceding section it was explained that the effect of disease upon any particular insect species is manifested by its effect upon the population density of that species and upon the biological equilibrium concerned with the existence of that species in nature. Disease undoubtedly constitutes an important part of the environmental resistance which opposes the biotic potential (reproductive potential and survival potential) of any particular insect species. Unfortunately, virtually no accurate experimental data relative to the environmental resistance (as afforded by disease) and population density, or for that matter to the fundamental role of disease in insect ecology, are available.[1]

In the absence of experimental data, however, certain generalizations and dogmas have emerged which apply to disease as well as to insect parasites and predators. At one time there were at least two distinct schools of thought with regard to the problem of biological equilibrium: the climatic school and the biological school. A third, the school of mathematical analysis, is sometimes considered as distinct from the other two. A leading proponent of the climatic school was Bodenheimer (1928), who believed that such factors as parasites, predators, and disease were much less important than was climate in regulating the population density of insects. Bodenheimer (1938) later modified his views and acknowledged that the part played by biotic factors had been underestimated. This change of opinion was probably influenced by the vigorous arguments of the biological school, to which H. S. Smith is a leading contributor. The biological school made a distinction between density-dependent factors (*e.g.*, parasites and diseases) and density-independent factors (*e.g.*, climate, although not always). On the basis of theoretical considerations as well as practical illustrations, Smith (1935) and others maintain that parasites, predators, and diseases are of great importance in the determination of the average population densities and that therefore an attempt to lower these densities by the introduction of such organisms is based upon a sound scientific foundation. The reason, according to Smith, that the possibilities of lowering these densities by biological methods are limited is

[1] The reader interested in certain of the theoretical aspects of biological control as they relate to the determination of and the dynamics of population densities is urged to consult such publications as those by Bodenheimer (1928, 1938), Thompson (1930), Chapman (1931), Nicholson (1933), Gause (1934), and Smith (1935). The differences in the viewpoints of the "climatic school" and the "biological school" are brought out in these writings. Certain of the well-known works of Raymond Pearl, and other biostatisticians, are also applicable to the subject of biological control.

not because the underlying principles are unsound but because in many cases the insect to be controlled does not have in its native environment a density-dependent mortality factor that can be successfully transported to and established in the new habitat.

To be sure, there are numerous aspects of insect ecology that should be considered when dealing with the diseases of insects, and such considerations have been kept in mind throughout the writing of this book. The significance of population studies has been mentioned here primarily to point out that the biotic factors of an insect's environment are a very important part of the ecology of insect life. This point is emphasized when we consider an epizootic that results in the economic control of a pest, but it is also important during nonepizootic periods as well. The regular or periodic natural occurrence of disease among insect populations constitutes an important ecological factor regardless of whether one is considering it from the standpoint of the practicability of biological control or from the standpoint of insect ecology generally.

BRIEF RÉSUMÉ OF SOME PAST ATTEMPTS TO CONTROL INSECTS BY THE ARTIFICIAL DISTRIBUTION OF MICROORGANISMS

Although the roles of different species of microorganisms in the control of certain insect pests have been mentioned consistently throughout the pages of this volume, it would seem advantageous at this point to recapitulate to some extent what has been said and to attempt a few generalizations. This may best be accomplished perhaps by considering briefly each of the major groups of organisms concerned (bacteria, fungi, viruses, protozoa, and nematodes) more or less in a chronological order and in relation to their effectiveness as control agents.

Bacteria

In the past much of the experience with man's use of bacteria in the control of insects has been similar to that obtained during the latter part of the nineteenth century, when efforts were made to control mice and rats by initiating dysenteric diseases among them (see Danysz, 1895). At first great success was reported; then the enthusiasm waned, and finally the method was abandoned. So it was with the first well-publicized instance in which bacteria were used in an attempt to control insects. We refer to the case of the so-called *Coccobacillus acridiorum* and the dysenteric disease it causes among grasshoppers. Let us consider certain aspects of this instance in some detail.

Coccobacillus acridiorum d'Her. (*Aerobacter aerogenes* var. *acridiorum* (d'Her.)) was first isolated by d'Herelle in Yucatan, Mexico, from locusts of the genus *Schistocerca*. While in Mexico, d'Herelle noticed a

heavy mortality occurring in the migratory locusts that had arrived from Guatemala. In 1910 and 1911 the epizootic occurred so extensively that by 1912 the number of locusts was reduced to the extent that no invasion into Mexico occurred. By artificially distributing the bacteria, d'Herelle apparently was successful in combating plagues of *Schistocerca* not only in Mexico but in Argentina and Tunisia as well, although in the latter country mechanical methods were employed concurrently. Since d'Herelle's early successes, some workers have been able to confirm his results, while others have failed completely. The latter appear to have been in the majority, and their pessimism apparently has killed all interest in further consideration of the organism as a means of biological control.

Now, it is important that we ascertain the basis of these contradictory results and claims. This is not easy to do in the case we are discussing; but by close inspection of the numerous papers on the bacterium concerned several pertinent and highly significant facts are revealed that are frequently overlooked by critics of this method of biological control (see also Chap. 9).

In the first place, there seems to be a difference in the susceptibility of different locusts to the disease. The bacterium generally appears to be more effective against locusts of the genus *Schistocerca* than against other genera of the Locustidae, and also more effective against those species which are cannibalistic and migratory in habit. Some grasshoppers are almost completely resistant to the bacterium when the latter is introduced perorally. Yet the method has been condemned by workers using it against grasshoppers only distantly related to *Schistocerca*, and against those which are noncannibalistic and nonmigratory. Secondly, and as was pointed out in Chap. 9, it has been found that several strains of "*Coccobacillus acridiorum*" exist, not all of which are equally pathogenic. Strains exist which are closely related but which nevertheless are distinct and cannot be considered the same as the organism isolated and used by d'Herelle. Indeed Glaser (1918) discovered that some of the cultures circulating under the name "*Coccobacillus acridiorum*" were of entirely different species or subspecies. Then too the typical strains of the bacterium rapidly lose their virulence on artificial culture media unless periodically passed through susceptible locusts. Thirdly, strains of low virulence and strains of closely related bacteria are frequently found normally present in locusts, and these may immunize the insects against the fully virulent *Coccobacillus acridiorum*.

Considering the little attention paid these intrinsic and variable factors, it is little wonder that inconsistent results were obtained. This is not to say that d'Herelle's methods were all he claimed them to be.

It is entirely likely that some of his observations were favored by natural outbreaks of disease coincident with his artificial distribution of the bacterium. Furthermore, in the absence of adequate controls, it is possible that d'Herelle himself became somewhat overenthusiastic and drew conclusions too sweeping in their scope. Certainly the method has not lived up to the original expectations. Nevertheless some of the adverse criticism and condemnation of the method appear to have been unreasoned or based on results obtained with considerable disregard for the ordinary principles of bacteriology and of host susceptibility and resistance, which d'Herelle insisted were essential for the successful execution of the method. Also shown clearly is the need for more basic research into the biological relationships involved in the diseases of insects.

D'Herelle's coccobacillus is not the only bacterium that has been tried and eventually found wanting. The literature contains accounts of numerous instances in which more or less superficial attempts have been made to control insect pests with bacteria. Some efforts have consisted essentially of finding a few diseased or dead insects in the field or laboratory, culturing them, and scattering the bacteria obtained about experimental plots in a haphazard manner. The reasons for the discouraging results obtained with such dilettant procedures are obvious. On the other hand, some attempts to use bacteria as control agents have failed in spite of a background of some fundamental research and a most careful and well-planned method of executing the field trials. This is to be expected, since it would be illogical to assume that every bacterial disease of an insect would have wide-scale applications in the field.

It is interesting to follow the experimental work of those investigators who, during the 1920's, strove to find some way by which bacteria could be used to control the European corn borer, *Pyrausta nubilalis* (Hbn.). If we are to judge from the published accounts of these investigations, certain bacteria are among the most effective agents known for controlling the corn borer. For some reason, which the writer has never been able to ascertain with certainty, these successful reports cease shortly after 1930, and one is left in the dark as to why the bacterial method of control did not blossom into general use. The success that was obtained merits the following brief inspection.

The first requirement for instituting a microbial attack against the corn borer was to obtain bacteria pathogenic to the insect. Among the hundreds of larvae collected in nature were large numbers of diseased or dead individuals from which quite an array of bacteria were isolated. Thus Metalnikov and Chorine (1928*a*) isolated four species; Chorine (1929*a*,*b*) himself isolated five species; Metalnikov, Ermolaev, and Skobaltzyn

(1930) described several strains each of three species; and Husz (1928) as well as others, employed a bacterium (*Bacillus thuringiensis* Berl.) originally isolated from diseased *Ephestia kühniella* Zell.

Most of the bacteria isolated by these workers were made up into aqueous suspensions or into dusts, and sprayed on corn plants in experimental plots. Metalnikov and his group made their field tests in Yugoslavia. Of the four bacteria (*Coccobacillus ellingeri* M. & C., *Bacterium* [*Bacillus*] *galleriae* No. 2 M. & C., *Bacterium* [*Bacillus*] *canadensis* Chor., and *Bacterium* [*Bacillus*] *thuringiensis* (Berl.)) tested in 1929, *Bacillus thuringiensis* was the most effective in causing a significant mortality of cornborer larvae. Whereas check plants, which had not been sprayed with the bacillus, contained an average of 16.7 borers per corn stalk, the plants of two series sprayed with *B. thuringiensis* averaged only 1.3 and 1.4 undersized larvae per stalk. There were corresponding differences in the number and size of the corn ears from the treated and untreated plots. In a continuation of these experiments Metalnikov, Hergula, and Strail, in 1930, reported mortalities of from 96.8 to 99.2 per cent in treated plots as compared with mortalities of from 81.7 to 87.5 per cent in nontreated check plots. These results were obtained under what these authors considered to be very unfavorable weather conditions. The bacteria used in this case included, in addition to *B. thuringiensis*, two other sporeformers, *Bacterium* [*Bacillus*] *pyrenei* Metal., and *Bacterium* [*Bacillus*] *cazaubon* Metal.

The favorable results obtained by Metalnikov and his associates were supported by equally encouraging results obtained in Hungary by Husz (1928, 1929, 1930, 1931), using *Bacillus thuringiensis* in dusts and sprays. This investigator reports that the bacterial treatment reduced the cornborer infestation from 36 to 14 per cent. He concluded that bacteria may be applied successfully in the fight against the corn borer.

Apparently uninterested in continuing the bacterial warfare against the corn borer on an economically significant scale, Metalnikov (1930) next turned his attention to the microbial control of the gypsy moth, *Porthetria dispar* (Linn.), and other destructive Lepidoptera. On an experimental basis he found that the same bacteria that had been successfully employed against the corn borer were likewise effective in destroying the gypsy-moth caterpillar. Mortalities of 100 per cent were obtained with sprays as well as with dusts containing the bacteria (*B. thuringiensis*, *B. cazaubon*, and *B. pyrenei*). Then, in collaboration with his son, who also worked alone (Metalnikov, Jr., 1933), Metalnikov (Metalnikov and Metalnikov, 1932, 1933) experimented with some of the bacteria pathogenic to corn-borer larvae using them against the pink bollworm, *Pectinophora gossypiella* (Saund.), and against *Prodenia litura* F. In addition, bacteria

were isolated from these insects and were used in their experiments. In Egyptian cotton fields heavily infested with the pink bollworm, the Metalnikovs sprayed mixtures of molasses, water, and spores (*B. ephestiae*, *B. cazaubon*, and *B. gelechiae*) on the plants two to four times at regular intervals at a rate of 196 gallons or less per acre (0.25 ounce of spore powder per 2.5 gallons of mixture). The infestation on the bacterial-treated plants was reduced by as much as 40 to 50 per cent. Plots treated with arsenical spray showed a reduction of only 18 per cent. Later Metalnikov (1937) claimed a mortality of 100 per cent in experiments using suspensions of bacterial spores against the bollworm. In France, *Sparganothis pilleriana* Schiff. on grape vines treated twice with spore suspensions were reduced in numbers to 14.4 per cent of those on untreated vines. Increased yields of grapes were obtained from the treated vineyards over those from the untreated controls (Metalnikov, 1940). In southern France, complete control of *Clysia ambiguella* Hbn. was obtained in 24 hours by such treatment. Such results encouraged the commercial preparation of bacterial spores which were sold and distributed in France. One of the last microbial enterprises attempted by Metalnikov (1942) was that of using bacterial spore dust against *Ephestia elutella* (Hbn.), which he found infesting flour. He considered it probable that several of the insect pests of flour may be successfully controlled by the proper use of spore dust that retained its effectiveness for several years.

There is little doubt that one of the most successful of all attempts to control an insect by microbial means is that achieved in the control of the Japanese beetle, *Popillia japonica* Newm., through the use of the bacteria (*Bacillus popilliae* Dutky and *B. lentimorbus* Dutky) causing the milky diseases of this insect. In order to supplement the natural spread and to accelerate the build-up of these bacteria, the U.S.D.A.,[1] as well as several state agencies, instituted an extensive program for the distribution of *B. popilliae*. This colonization program was begun in 1939 and is still in progress (1949). From 1939 to the end of 1948, a total of 151,559 pounds of spore dust was used to treat 90,791 sites covering 73,618 acres in 12 states and the District of Columbia. In addition, distribution by private individuals on their own property has been made possible by the licensed production of spore dust by several commercial concerns that market the product under their own trade names.

The over-all effects or detailed results of this distribution program have yet to be ascertained. In general, however, marked reductions in the Japanese-beetle-grub population have occurred in treated areas, especially

[1] In November, 1947, the workers on milky disease at the Japanese Beetle Laboratory at Moorestown, New Jersey, were presented an award for superior service by N. E. Dodd, Undersecretary of the U.S. Department of Agriculture.

after the 2 or 3 years allowed for the natural spread have elapsed. Populations as high as 44 grubs per square foot have been reduced to less than 5 per square foot, 4 years after an intensive milky-disease treatment. Present indications are that the milky disease (naturally and artificially induced) constitutes one of the most effective means of bringing about a gradual reduction in the Japanese-beetle population of northeastern United States.

On the basis of the facts brought out in the foregoing paragraphs, it is apparent that the use of bacteria in the control of insect pests has not had a really thorough or extensive study. The success of the use of the milky diseases against the Japanese beetle is indicative of what might be accomplished with certain entomogenous bacteria when their use is based on careful, thorough, fundamental, scientific research supported by adequate funds and personnel.

With regard to the bacteria, it appears that with our present knowledge, at least two generalizations may be safely made at this time: (1) sporeforming bacteria lend themselves to use as control agents more satisfactorily than do nonsporeforming bacteria; (2) strictly entomogenous parasites, *i.e.*, bacteria pathogenic principally for insects, are more effective control agents than are pathogens for other animals or than are ordinary saprophytic bacteria.

The first of these generalizations is almost self-evident when the great resistance of bacterial spores to adverse conditions is considered. Such a characteristic enables the bacterium to survive for relatively long periods of time outside its living host, free in nature. Environmental factors such as adverse temperatures and humidities which destroy most nonsporeforming bacteria are withstood by sporeformers. Furthermore the resistant spore more readily lends itself to the production of dusts and other dry preparations, facilitating easy distribution and marketing. This is not to say that nonsporeforming bacteria are incapable of causing widespread disease among insects. The natural outbreaks of dysenteries caused by "*Coccobacillus acridiorum*" attest to the fact that nature can use nonsporeforming bacteria very effectively. In the hands of man, however, the story has so far been quite different. It therefore appears justifiable to assert that, until man has learned how to handle the nonsporeforming bacteria more effectively, it behooves him to concentrate on the sporeformers, now mostly in the genus *Bacillus*.

Ever since the early work of Paillot, Metalnikov, and others who conducted infectivity experiments on insects, it has been known that a large enough dose of almost any readily cultivable bacterium would cause infection, and usually death, when inoculated directly into the body cavity of the insect. Thus common saprophytes, such as the coliforms and

common soil-inhabiting bacilli, then introduced into the hemocoele of an insect, regularly produce a fatal septicemia. Such is not commonly the case, however, when these bacteria are fed to the insect. The oral introduction of most of these bacteria into the digestive tract of the insect usually produces no untoward reactions. Now practical and effective control of insects in the field certainly cannot be brought about by the direct inoculation of cultures into the body cavity of the insects. We must therefore select bacteria that in themselves have the power to invade the body of the arthropods. Contrary to what one might at first think, the fastidious bacteria that invade the tissues of man and other vertebrates causing infection and death, have, with few exceptions, relatively little power to invade and infect insects. Thus the dread tubercle bacillus, the pneumococcus, and others are relatively noninfectious for most insects. On the other hand, those entomogenous bacteria such as *Bacillus larvae* and *Bacillus popilliae*, which are known to parasitize insects exclusively, are capable of causing considerable degrees of morbidity and mortality among insects. These facts lead us to the warranted assumption that the most effective bacterial agents from the control standpoint are probably to be found among those bacteria which are natural parasites or pathogens of insects.

Fungi

Just who first conceived the idea of using entomogenous fungi to combat insect pests is difficult to determine. In any case one of the most interesting points at which to pick up the thread of these early conjectures is in Europe at a meeting of an association of naturalists in 1861. Among the speakers at this meeting was a biologist named Bail, who exhibited a mold growing on a mash that had been sown with "the fungus of the housefly." Particularly memorable to those who attended this demonstration was the reputedly fine beer brewed from this mash, and a cake baked with the yeast fungus which Bail believed to be produced from the mold sowed on the mash. Bail maintained that the mold and the yeast fungus were capable of killing insects (flies, mosquitoes, caterpillars) brought in contact with the mash. To be sure, such beneficial insects as the silkworm and the honeybee were, at this time, known to suffer from infection by fungi, but little attention had been paid to these infections in destructive insects. Naturally occurring epizootics had been observed among certain flies, gnats, and caterpillars, but practically no progress had been made toward developing ways of distributing the fungi artificially.

Among those impressed by Bail's work was the American entomologist H. A. Hagèn, who in 1879 advocated the use of "the yeast fungus" against noxious insects. This followed earlier suggestions by Pasteur (see Prentiss,

1880) and by LeConte (1874), who recommended the careful study of the fungous diseases of insects and their possible use in control. Hagen's proposal was given several trials, with marked success claimed in one instance. Although most of these men thought they were working with the true housefly fungus, one is impressed with the probability that they were really dealing with adventitious yeasts or fungi. This supposition seems warranted for several reasons, not the least of which is Hagen's conclusion that "the fungus of the house-fly works as well as yeast for baking and brewing purposes."

During the same year (1879) in which Hagen made his proposals, Metchnikoff reported the results of experiments in which he infected insects by artificial means. This famous biologist mixed the spores of the green-muscardine fungus (*Metarrhizium anisopliae* (Metch.)) with soil in a container and placed in this soil the healthy larvae of the wheat cockchafer (*Anisoplia austriaca* Hbst.), which subsequently became infected and died. Metchnikoff (1880) also found the sugar-beet curculio, *Cleonus punctiventris* Germ., to be susceptible to the same fungus. It was to combat this insect that Krassilstschik, in 1886 and 1888 in his laboratory at Smela near Kieff, successfully produced spores of the fungus in quantities sufficient for field distribution. He obtained a mortality of 50 to 80 per cent in his experimental plots. Brongniart (1888) recommended the scattering of entomophthoraceous fungi among flies and other common insects as a means of inexpensive control. Similar recommendations were made by Künckel de Herculais and Langlois (1891) with regard to grasshoppers. In 1892 the physiologist Franz Tangl (1893) attempted to use the white-muscardine fungus, *Beauveria bassiana* (Bals.), against caterpillars of the nun moth, *Lymantria monacha* Linn. His laboratory experiments succeeded, but in nature the trees sprayed with spore suspensions gave negative results, there being no substantial reduction in the number of caterpillars. About this same time von Tubeuf conducted similar experiments using *Cordyceps militaris* (Lk.), with negative results.

Metchnikoff's and Krassilstschik's work on *Metarrhizium anisopliae*, the green-muscardine fungus, as well as subsequent observations of its occurrence in nature, stimulated other workers to investigate its possibilities as a control agent. Projects were undertaken in Java, Samoa, Hawaii, Trinidad, Puerto Rico, and other regions of the world. As reviewed by Stevenson (1918) some of these attempts were rewarded with encouraging success, while others failed completely. The most promising results were obtained in Trinidad against the froghopper, *Tomaspis varia*, on sugar cane dusted with a mixture of flour and spores. A sufficiently large number of insects were killed to justify the erection of spore-producing plants on a number of the sugar estates. In general,

however, it has been found that with most insects the fungus is so dependent upon the proper conditions of temperature and humidity as to make its artificial distribution impractical except when optimum conditions prevail and when the quantity of indigenous fungus in an area is low. The effectiveness of naturally occurring epizootics continues to be significant in certain areas under favorable climatic conditions.

In the United States the first real impetus to widespread interest in the use of fungi as agents to control insects came with the work initiated by Forbes (1882) in Illinois, and by Snow (1888) in Kansas on the control of the chinch bug, *Blissus leucopterus* (Say), by means of the white fungus, *Beauveria globulifera* (Speg.). These men, along with others in Minnesota, Iowa, Ohio, and elsewhere, observed extensive natural outbreaks of the disease which aided greatly, and still do, in the control of the chinch bug in the presence of favorable climatic conditions. It was only natural that the possibilities of increasing its effectiveness through the artificial distribution of the fungus invited investigation, not only in the United States but abroad (*e.g.*, Trabut, 1898*a*,*b*, 1899). Since the details of the chinch-bug studies have already been described in Chap. 10, only those aspects of the subject pertinent to our present discussion need be mentioned here.

The first attempt at the artificial distribution of the chinch-bug fungus was made in Minnesota by Lugger (1888), who scattered diseased bugs about the fields. He was apparently successful in initiating an epizootic, but there is reason to believe that the spores were already present in the area concerned and that these may have given rise to the outbreak. It was not long, however, before other investigators studied the feasibility of distributing the fungus artificially. In 1891 the Kansas state legislature established an experiment station at the University of Kansas for the purpose of propagating and distributing the fungus. This work was placed under the direction of F. H. Snow, who did much to awaken entomologists, as well as farmers, to the potentialities of this type of biological control. The actual results of Snow's distribution program, as reported by field observers, were almost equally divided between "successful" and "unsuccessful" control. In addition, as we have described elsewhere, several pertinent fundamental facts relating to fungous diseases in nature were brought out by this work. It slowly became apparent, however, that it was no simple matter to initiate and maintain an epizootic in nature by artificial means. Certain essential and intrinsic factors were at work that proved difficult to understand or to utilize. Lugger abandoned his attempts by 1902, and a similar desertion of the method followed in Illinois, Nebraska, Missouri, Ohio, Oklahoma, and finally in Kansas.

One of the principal reasons why the artificial use of the chinch-bug fungus was abandoned can be ascribed to the report published by Billings

and Glenn in 1911. The important conclusion reached by these investigators was that in fields where the natural presence of the fungus is plainly evident, its effect on the chinch bug cannot be accelerated to any appreciable degree by the artificial introduction of spores. Furthermore in fields where the fungus is not in evidence spores introduced artificially have no measurable effect; the apparent absence of the fungus among chinch bugs in the field is evidence of unfavorable conditions rather than of the lack of fungous spores. Since the data gathered by Billings and Glenn indicated that nothing could be gained by the artificial dissemination of the fungous spores, it appears that the authorities were justified in abandoning their distribution programs until more knowledge was at hand concerning the role played by the various climatological and other extrinsic factors. One should, at this point, be cautious about drawing any broad conclusion relating to fungous diseases generally. It should be remembered that the conclusions reached by Billings and Glenn apply specifically to the chinch bug, to the particular fungus with which they were concerned, and to the general area in which they worked. As has been pointed out by Fawcett (1944),

> The failure to attain increased mortality by artificial distribution of this fungus has been often cited as an example of what may be expected from entomogenous fungi. It may be pointed out that this result might have been expected from a fungus of this kind which has many hosts and which produces such abundance of windborne spores that may become widespread and reach a "saturation point" under most all conditions suitable for infection. What was found with this fungus is not necessarily a criterion by which to judge possibilities in other fungi.

The latest and most authoritative consensus relative to the effectiveness of the chinch-bug fungus appears to be that if it is a true parasite, it is probably the most destructive natural enemy of the chinch bug; that it is generally present in fields throughout the country; that its effectiveness is dependent largely upon the weather; and that since it has been shown that the spores of the fungus are present wherever the bugs are common, its artificial dissemination as a control measure is unnecessary.

While the experiments with the chinch-bug fungus were under way, the practical use of other entomogenous fungi was being considered in various parts of the world. In Natal, South Africa, grasshoppers were found dying in large numbers from a fungous disease. Although the natural infection may actually have been caused by *Empusa grylli* (Fres.), a *Mucor* commonly found on dead organic matter was isolated and distributed as being the causative agent. In any event, this *Mucor* was apparently an insect killer, since favorable reports were made of its use in both South Africa and Australia. About this same time (1897), a *Sporotrichum*

(*Beauveria*) was found destroying considerable numbers of grasshoppers in Argentina (and such outbreaks still occur there; Marchionatto, 1934). These reports induced L. O. Howard (1902), in the United States, to investigate the possible use of fungi in the control of grasshoppers in this country. He obtained cultures of the South African *Mucor* and had the fungus distributed in various parts of the United States. The reports from its users varied from enthusiastic success to complete failure. They indicated that the hopes of that day relative to the control of grasshoppers by fungous diseases had been placed too high and that these microorganisms were not the complete answer to grasshopper control. Howard concluded, however, that under favorable conditions some good results from the distribution of the grasshopper fungi had been obtained. Favorable results continue to be reported, however, indicating that further investigation is needed. Petkov (1939), for example, reports an 83 per cent kill of locusts in Bulgaria after spores of *Empusa grylli* were scattered on plants.

Soil insects were also being found subject to attack by fungi about this time, as is evidenced by the observations of Giard (1891,*a*,*b*,*c*,*d*.) and others, who employed *Beauveria densa* (Lk.) against white grubs and cockchafers with varying success. Although this fungus is known to have a considerable number of hosts, little experimentation has been given to it as a control agent since the early trials of European workers. Further study also appears to be called for in the case of *Sorosporella uvella* (Krass.), a fungous parasite of noctuid larvae (Speare, 1920).

In 1912 Speare and Colley published a very encouraging report on the artificial propagation and use of the brown-tail fungus, *Entomophthora aulicae* (Reich.), against larvae of the brown-tail moth, *Nygmia phaeorrhoea* (Donov.). They considered the fungus to be an effective means of destroying this insect and obtained results in which the introduced disease could be depended on to kill from 60 to 100 per cent of the caterpillars in the areas concerned. As in most other attempts to use the difficult-to-cultivate Entomophthoraceae against insects, these investigators used infected insects as foci of infection in order to disseminate the fungus. Similar methods have been used in attempts to disseminate artificially such entomophthoraceous fungi as *Empusa muscae* Cohn and *E. sciarae* Olive against various Diptera. A similar method has been used by Dustan (1924*a*) in disseminating *Empusa erupta* Dustan against the green apple bug in Canada, where the fungus is at times an important factor in the natural control of this insect.

In the United States, the climax to this early wave of popular interest in the use of entomogenous fungi in the control of insect pests came with the work in Florida (and in the West Indies; South, 1910) with the fungi

attacking whiteflies and scale insects (see also Chap. 10). The usual story of conflicting opinions as to the efficacy of this method of control prevailed. That under favorable climatic conditions large numbers of these insects were destroyed by the natural occurrence of fungi was not doubted, but that man could aid in the distribution and effectiveness of the microorganisms was strongly debated. Berger (1910, 1921, 1932) was convinced that the natural mortality of whitefly nymphs could be increased markedly by spraying infested orchards at the proper season (moist season of summer) with fungous spores. Morrill and Back (1912), on the other hand, contended that the fungi could not be depended on to give satisfactory results and that chemical remedies should be relied on instead. They did, however, mention that, under certain circumstances, such as in citrus groves located in low-lying hammocks where the use of insecticides would be impractical, fungous parasites may be used to advantage. As has been pointed out by Fawcett (1944), the difference in these results might be that Morrill and Back experimented at a period of the approximate saturation point of spores for infection, while Berger probably experimented at periods of unsaturation or of lag in possible infection for prevailing conditions. A similar statement may apply to the contradictory results obtained in the case of scale insects and the attempts to enhance their control through the agency of fungi.

Despite the differences of opinion that prevailed, the interest of farmers and entomologist alike in the practical use of fungi against citrus pests was maintained. Efforts to distribute the whitefly fungi artificially throughout the citrus-producing areas of Florida were made by the Florida Experiment Station and by private agencies that offered the fungi for sale. Growers usually obtained scale fungi, which were not produced commercially, from neighboring groves. Orchardists were convinced that it was to their advantage to have the fungi, in adequate numbers, in their groves whether introduced naturally or artificially. The conviction was supported by the advice of the Entomologist of the Florida Experiment Station (Watson, 1923), who asserted that it is very important that the grower have a good supply of these fungi in his grove and that if they are not already present in abundance, "it will pay him to make a particular effort to introduce them." Citrus growers in California also became interested in the role of fungi in the control of citrus pests. Climatic conditions unfavorable for the growth of these entomogenous fungi kept the interest somewhat subdued. Nevertheless efforts to convince the growers of the efficacy of such fungi and attempts to market cultures were made by private individuals, and for interesting and rather amusing reading on the subject the reader is referred to an article prepared by Woodbridge in the *California Cultivator* for February 18, 1915.

The final word on this matter remains to be spoken, however, and the true pathogenicity of some of the fungi found on scale insects and whiteflies remains to be determined. Such suspicions relating to the efficacy of these fungi and the early claims made for them are to a considerable extent sustained by the work of Holloway and Young (1943, 1948) on the purple scale, which, in many respects, supports the conclusion of Morrill and Back (1912) on whiteflies. While studying the influence of fungicidal sprays on entomogenous fungi infecting the purple scale in Florida, these men obtained data showing that the scarcity or abundance of fungi does not influence the rate of total mortality of scale insects. Furthermore, despite the claims of some earlier workers, no abnormal increase in scale insects appears to be associated with the fungicidal properties of the sprays, but increases are instead associated with the residues from the applied materials. Examination of the data obtained by Holloway and Young reveals a strong indication that mortality of the scale insects during Florida's rainy season, the time when the entomogenous fungi flourish, is associated with the wet weather rather than due directly to the fungi. There is an implication in their data to the effect that certain entomogenous fungi do not invade the living healthy insect but attack only those which have been weakened or made unhealthy by other influences associated with wet weather or with certain unknown extrinsic factors. Such a conception is reminiscent of views held more than 100 years ago when certain observers (see Gray, 1858, page 19) expressed the belief that the development of entomogenous fungi "does not depend altogether on being nourished by warmth and moisture . . . , but rather on the insect becoming sickly and feeble by the effect of heavy rains that fall at stated periods in the intertropical regions, or from the extremely humid seasons which prevail occasionally during certain months of the year in most extratropical countries." More recently Fisher (1948) has reported that while the endoparasitic fungus *Myiophagus* sp. does cause disease in purple scale all attempts to inoculate red and purple scales with *Sphaerostilbe*, *Nectria*, and *Podonectria* fungi have failed. Considerably more study is necessary before the conflicting views concerning this particular group of entomogenous fungi can be resolved. The data and statistics of Morrill and Back (1912), Osburn and Spencer (1938), Holloway and Young (1943), Fisher (1947, 1948), and Holloway (1949) certainly present sufficient reason for further investigations and for a revaluation of some of the results obtained by earlier workers. An attempt to reconcile the various opinions has been made by Fawcett (1944), who suggests that there "is a complex of possible fluctuating factors that need to be unscrambled by experiments with controlled conditions for the insects, for the fungi themselves, and for the complex fungus-insect relationship, before it can be

decided what part is played by the deposits or residues from applied materials, by nutrition of the tree and thereby nutrition of the insects, and by the parasitic organisms."

The European corn borer, *Pyrausta nubilalis* (Hbn.), is another insect that has received considerable study from the standpoint of its fungous parasites. Most of the interest shown in the possibility of controlling this destructive pest by means of fungi stems from the investigations of the same men who gave so much consideration to the use of bacteria against the same insect. This work started off with Metalnikov and Toumanoff's (1928) infection experiments in which they found the corn-borer larvae to be very susceptible to *Aspergillus flavus* Link, *Beauveria bassiana* (Bals.), and *Spicaria farinosa* (Fres.), and to a lesser extent, *Sterigmatocystis nigra* Teigh. (see also Toumanoff, 1933). Similar studies were made by Wallengren and Johansson (1929) using the green-muscardine fungus, *Metarrhizium anisopliae* (Metch.). Wallengren (1930) found that dusting the corn plants with the conidia of this fungus protected them completely from corn-borer attacks. Actual counts revealed a mortality of 99.3 per cent. Similar results were obtained by Hergula (1930, 1931), who conducted a series of field experiments in which the *Metarrhizium* spores were mixed with such vehicles as starch, dextrin, and tragacanth. He (1930) records a mortality of 99 per cent on spore-dusted plants as compared with 85.6 per cent on untreated plants. Of the dusted plants, 25.1 per cent contained borers, while 98 per cent of the check plants were infested. On the basis of these experiments Hergula believes that theoretically the use of this fungus offers an effective method of combating the corn borer. His expectations, however, remain to be fulfilled as far as the practical application is concerned.

In the United States a laboratory outbreak of *Beauveria bassiana* (Bals.) in corn-borer larvae imported from Manchuria caused Lefebvre (1931*a*,*b*) to study this fungus in relation to its host. These studies were enlarged when Bartlett and Lefebvre (1934) conducted field experiments to determine if the fungus could be established in the corn borer in nature. They obtained indications that this would probably be the case and observed that the larvae in the field were readily susceptible to attack by the fungus when the spores were mixed with flour as a carrier. Further field experiments were conducted in Canada by Stirrett, Beall, and Timonin (1937), and by Beall, Stirrett, and Conners (1939), who state that the time of application of the spores is of greatest importance in the effectiveness of the fungus; the rate at which the spores are applied did not seem to be of great importance. In both 1936 and 1937 they obtained a control of 60 to 70 per cent. *B. bassiana* appears to be responsible for natural epizootics in the case of several other economically important insects such

as the codling moth. Furthermore populations of the Mexican bean beetle in the state of New York have been effectively controlled with the fungus. Dresner (1947) found the field use of *B. bassiana* to be more effective than similar use of rotenone against this insect.

In many respects analogous to the work with the fungous parasites of the corn borer has been the work in France with the fungi attacking insect pests of vineyards. Early reports using *Spicaria farinosa* (Fres.) against such grapevine pests as *Polychrosis botrana* Schiff. and *Clysia ambiguella* Hüb. were encouraging, particularly in those regions where the vine stocks were buried during the winter. Such practices also favored natural outbreaks of the infection, 85 to 95 per cent mortalities being recorded. Some French workers report the obtaining of excellent results merely by scattering spore suspensions over the infested vines; others report only failure.

Out of the studies and controversies concerned with the control value of entomogenous fungi, at least one definite fact stands out clear and indisputable: Entomogenous fungi, in nature and without any help from man, cause a regular and tremendous mortality of many insect pests in many parts of the world and do, in fact, constitute an efficient and extremely important natural control factor. Accordingly, entomogenous fungi are of great economic importance in insect control, even though man has not yet learned how to use them artificially in most instances. This realization was held by many of the early students of entomogenous fungi (see review by Picard, 1913). Few, however, have brought this out as clearly as did Speare (1922) during his work on the natural control of the citrus mealybug in Florida through the activity of *Entomophthora fumosa* Speare (see page 337). He went so far as to say that "entomogenous fungi are worth millions of dollars to the citrus industry. Owing to their excellent work, oranges and grapefruit are grown at a profit in many parts of the State where no money whatsoever is spent on artificial remedial measures." It must of course be recognized that entomogenous fungi are more effective as natural enemies in those areas in which the conditions of temperature, humidity, and the like favor their growth and development. In areas where such conditions do not prevail, the activities of the fungi are limited to periods, probably sporadic in occurrence, in which favorable conditions do occur.

The artificial use of entomogenous fungi is dependent upon man's understanding of the numerous natural factors involved. Not only must he know when to apply the fungus with respect to the temperature and humidity, but he must have a thorough understanding of the saturation point, climatological influences, rate and time of application, and numerous other intrinsic factors relating to insect populations.

Viruses

In spite of the fact that viruses cause widespread natural mortality among some of the most destructive lepidopterous and hymenopterous pests, man has been able to accomplish very little in the way of artificially distributing these viruses so as to enhance their control value. One of the prime reasons for this is undoubtedly the great difficulty that exists in producing viruses in large quantities. Since these agents cannot be grown on ordinary nonliving bacteriological media, more elaborate methods must be used for their cultivation. Only recently has the way to do this been opened. Attempts to redistribute virus-diseased insects as found in nature have not been practical. More productive methods of propagation must be found if wide-scale artificial distribution is to be accomplished.

Theoretically, at least two laboratory methods of producing viruses in large quantities exist: (1) tissue culture and (2) insectary production. It is obvious that any attempt to produce polyhedral viruses in mass quantities by the delicate tissue-culture methods used for experimental purposes would not be feasible. It is entirely possible that something comparable to the use of the fertile eggs of hens in the production of mammalian viruses would prove workable. The hen's egg has been found to be one of the greatest aids in the cultivation of many viruses and rickettsiae, since it is a veritable tissue medium enclosed within the microbial-free confines of the eggshell. So far, however, the most promising method of producing large quantities of insect virus such as would be necessary for field distribution has been through the expedient of rearing large numbers of the insect host in an insectary. In this way thousands of hosts may be reared, artificially exposed to the virus, and after becoming thoroughly infected, the hosts may be sacrificed in such a manner as to yield large quantities of the virus. A variation of this insectary method of acquiring large quantities of virus consists in collecting the host insects in the field in the early stages of infection and then holding them in the laboratory or insectary until they succumb, after which virus suspensions may be prepared from their dead bodies. Another variation has also been found practical. Healthy larvae may be collected by the thousands from heavily infested fields, then placed, along with the necessary food, in properly constructed barriers where, if the virus is latently present in them, the disease will soon affect all the larvae. They may also be infected by the introduction of a few diseased individuals or by spraying the food plants with a small quantity of virus suspension. After the insects die, they may be gathered from the barrier, made into a suspension, and then properly stored or refrigerated.

One of the first attempts to initiate artificially an outbreak that may

be considered with fair certainty to be a polyhedral virus disease was that reported by Lounsbury in 1913. Diseased and dead lucerne insects (*Colias electo* Linn., and *Heliothis "obtectus"*) and the food plants they had fouled were comminuted in a bucket of water and sprinkled over caterpillar-infested lucerne. Outbreaks of disease followed such applications, but there was reason to suppose that the disease might have occurred anyway. At any rate, it was concluded that the efficiency of this method of control could not be economically increased by the artificial dissemination of the infectious material. Lounsbury presumed that an epizootic could be initiated in a lucerne field merely by greatly overirrigating a part of the field where the caterpillars are abundant, an observation made several times since by other workers.

Evidence that a polyhedral virus may be introduced into new areas by spraying foliage with an aqueous extract of diseased larvae has been obtained by Balch and Bird (1944) working with the polyhedrosis of the European spruce sawfly, *Gilpinia hercyniae* (Htg.). According to Balch (1946), prior to the introduction of dried extracts of the diseased insects into Newfoundland, no diseased sawfly larvae had been observed on the island. Following this introduction, the disease became prevalent over considerable areas surrounding the points of liberation. Similar results have been obtained in certain forest areas of Europe, where the efforts to control nun-moth infestations by means of virus have been made.

Another example of the use of a virus to control populations of a destructive insect is provided by recent experiments along this line by Steinhaus and Thompson (1949) in the case of the alfalfa caterpillar, *Colias philodice eurytheme* Bdvl., in California. Large numbers of the insect were infected with the polyhedrosis virus in the laboratory. The infectious material was prepared for field distribution by triturating the caterpillars to a thick homogeneous suspension in a Waring blender. The material was then diluted 1 : 3 with distilled water, passed through cheesecloth to remove large particles, and a hemacytometer count made of the approximate number of polyhedral bodies in the suspension. The concentrated infectious material was taken into the field and then further diluted so that the polyhedral count of the final spray solution was between 50,000,000 and 100,000,000 per milliliter. The spray was directed uniformly over alfalfa plots from an ordinary 5-gallon back-pack hand sprayer. In general, the results of these experiments indicated the practical value of distributing virus material artificially to control the caterpillar. Populations of low as well as of high densities were checked and markedly reduced by means of the virus.

It is not improbable that in many localities the situation with respect to the natural outbreak of virus epizootics is similar to that in the case of

fungi; *i.e.*, the polyhedral virus may persist in the environment (soil, foliage) of the insect for considerable periods of time and give rise to disease when conditions of moisture, temperature, and population density are favorable.

Protozoa and Nematodes

The use of protozoa in the control of insect pests is in somewhat the same state as is that of the viruses. Very few entomogenous protozoa have been cultivated on artificial media, and hence it has been a difficult matter to produce them in large enough quantities for practical field distribution. Occasionally biologists have scattered the spores or cysts of protozoa about over experimental plots, but apparently no wide-scale programs have been undertaken. Taylor and King (1937), for example, collected from contaminated cages the feces of grasshoppers (*Melanoplus*) infected with an amoeba, *Malameba locustae* (K. & T.), and mixed this material with bran and molasses. This mixture was scattered along roads and fences over a small area. Examination of the grasshoppers in this area 8 weeks later showed a small but significant increase in the percentage infected.

Potentially one of the most important groups of protozoa from the biological-control standpoint is that of Microsporidia. The members of this order have fairly resistant spores, and many of them are very pathogenic for insects. Unfortunately no way of growing them, outside of their respective hosts, has been developed. It would appear to be entirely practical, however, to cultivate them in large numbers of insectary-reared hosts, as we have already suggested in the case of the virus diseases. Spore dusts of some microsporidia have been prepared in this manner.

What has been said of the past difficulties in using viruses and protozoa in biological control may, to a large extent, be said of the entomophilic nematodes. These animals have been reared apart from their hosts in only a few instances. Where this has been accomplished, considerable progress has been made in developing methods of artificially distributing the nematodes. The outstanding example of such an achievement is the case of *Neoaplectana glaseri* Steiner and its use against the Japanese beetle, *Popillia japonica* Newm. Glaser (1932), working in New Jersey, found that *Neoaplectana* could be established effectively in a region where it did not occur naturally. In 1935, Glaser and Farrell reported with greater certainty that the parasite could be introduced into Japanese-beetle-infested fields and become permanently established there, producing a high and effective mortality. Grub populations were reduced as much as 40 per cent. In 1940 Girth, McCoy, and Glaser described the results of 73 field tests using ensheathed nematodes. Depending on soil moisture,

nematode dosage, soil temperature, and density of the beetle population, the percentage of the total grub population parasitized ranged from 0.3 to 81.5 per cent. These authors also told of a nematode colonization program initiated in the state of New Jersey, colonies being established at 3½ mile intervals over the state in an attempt to produce a general distribution of the parasite over the area. The colonization program was subsequently completed in about 6 years. Sufficient time has not elapsed for an accurate appraisal of the effectiveness of the program, but there are indications that it may be one of the important factors that have been responsible for the decline of the Japanese-beetle population in New Jersey.

METHODS OF DISTRIBUTION

Entomogenous microorganisms, like all other forms of life, are dispersed naturally by any of a variety of agencies. The wind and the rain are undoubtedly important in this regard, but there are few specific or concrete data to provide information of the accuracy needed. Animals, such as birds, are known to be of significance in transporting infected insects from one point to another. Parasitic insects are capable of transmitting microorganisms from diseased to healthy individuals by means of their ovipositors. Direct contact with the infecting agent is undoubtedly one of the principal means by which transmission is effected. This contact is usually made through the agency of contaminated food; sometimes cannibalism is a factor. The migration and movements of the infected insect itself may also be important in natural dissemination of the pathogen.

Several methods of artificially distributing entomogenous microorganisms have been devised. Since no small part of the success obtained in the microbial control of insects lies with the manner in which the microorganisms are distributed, it seems worth while to consider here briefly a few of these methods. Some of the methods have been rather superficial and cumbersome; others have been quite efficient. In any case there is considerable room for developments along this line, particularly for wide-scale distribution.

Distribution of Diseased Insects. Perhaps one of the simplest and most obvious methods of artificially disseminating entomogenous microorganisms is the scattering of dead diseased insects or the redistribution of diseased but living insects. This method has been used with certain bacterial diseases of grasshoppers, as well as with certain fungous and virus diseases. In the former the simple expedient has been used of dipping fresh grasshoppers into suspensions of the bacterial culture and then releasing the insect again—eventually to become diseased and a source of infection to its fellows. In Trinidad the green-muscardine fungus was similarly distributed by catching froghoppers in tubes containing sporebearing

cultures and then, after a moment or two, releasing the insect again. The redistribution of living or dead insects infected with fungi has been successfully used in a number of instances, particularly when the fungus cannot be readily cultivated on artificial media. Dustan (1924*a*), for example, found one of the best methods of distributing *Empusa erupta* Dust. among green apple bugs in Canada to be the transference of diseased and freshly ruptured insects from one orchard to another. The transference of living infected apple bugs was also effective, since such insects would find shelter under the bark of trees where primary infection of the young nymphs would take place the following spring. Dustan (1924*b*) used a similar method to distribute *Entomophthora sphaerosperma* Fres. among the European apple sucker in Nova Scotia; so did Speare and Colley (1912) during their early work with *Entomophthora aulicae* (Reich) against the brown-tail moth in Massachusetts.

A noteworthy variation of the insect-transference method of distributing entomogenous fungi is that used by Florida workers in disseminating the fungi that parasitized whiteflies and scale insects. In addition to spore-spraying methods, these fungi have been applied to scale-infested trees by simply attaching leaves infested with infected insects on the tree to be treated in such a way as to allow the dews and rains to carry the spores over and among the healthy insects. Depending on the size of the tree, from one to a dozen pieces should be attached to each tree, although not every tree in the orchard need be so treated, since the fungus also spreads naturally. Such leaf-pinning or sprig-pinning methods are usually slow and not so effective as are spraying methods. Occasionally, however, they proved almost as effective as did the spraying methods, and they were less troublesome. Such was found to be the case by Dustan in his work on the control of the European apple sucker mentioned above. The fungus, *Entomophthora sphaerosperma*, usually appeared in an orchard about a week after the fungus-infested leaves were pinned. Once the fungus gained a foothold in a number of orchards, the insects themselves helped to spread the disease.

In the West Indies, South (1910) promoted the growth of entomogenous fungi by allowing trees attacked by scale insects to become covered with a fairly thick growth of Bengal beans. This method was applicable in windy places and in localities where the so-called "wet season" did not provide sufficient moisture. The fungi become more numerous in the damp sheltered areas under the beans.

Sprays and Dusts. Early workers with entomogenous fungi suspended the spores of these microorganisms in water and sprayed this mixture on plants infested with noxious insects. In Florida, for example, aqueous suspensions of whitefly and scale-insect fungi made from cultures or from

infected leaves (three or four dozen leaves to a gallon of water) were sprayed onto the citrus trees. An ordinary knapsack sprayer that had never contained a fungicide could be used for such operations. When enough material to spray the entire grove was not available, a limb or two of each tree was sprayed—enough to initiate the natural spread. Sprays have been similarly used to distribute several of the entomophthoraceous fungi as well as some of the Fungi Imperfecti. A few bacteria

Fig. 216. Application of milky-disease (*Bacillus popilliae* Dutky) spore dust to a grassed portion of an Army post. Distribution is made with 10- by 10-foot spacing. *(Courtesy of S. S. Easter, U.S. Army, Engineer Corps.)*

have also been applied in the form of sprays, such as those sporeformers (*Bacillus thuringiensis* Berl., *B. canadensis* (Chor.), *B. galleriae* (M. & C.), etc.) used in Europe in the control of the European corn borer.

Dusts, particularly spore dusts, have found rather wide application in attempts to control insect pests in the field. Such dusts have been used to disseminate both fungi and bacteria, and to a lesser extent viruses and protozoa. At first merely dried preparations of pure spores were used experimentally. The advantage of combining the spores with some carrier, however, soon became apparent. The carriers commonly used in insecticide dusts were found to be quite satisfactory for the preparation of spore dusts, talc being particularly applicable. In some of the early work with the chinch-bug fungus the spores were combined with dry earth, and the resulting mixture was dusted directly on the ground at the base of the plants where the insects congregate.

Spores of both the bacteria and the fungi used against the European

corn borer were frequently applied as dusts. The bacteria were usually grown on large surfaces of nutrient agar, washed off with a small quantity of distilled water or saline, added to talc, and allowed to dry in a drying oven. The dried preparation was pulverized in a mortar until a very fine powder was obtained. By adding water, this mixture could also be used as a spray. The fungous spores, produced on rice media or similar substrata, were mixed with either talc, starch, or wheat flour and applied in varying dosages. In the United States several other methods have been devised for culturing fungi in large quantities. McCoy and Carver (1941), for example, obtained large quantities of spores of *Beauveria bassiana* (Bals.) on moistened wheat bran and then purified them by an air separation and filtration method. They obtained a yield of about 22 grams of spores per pound of medium. Not finding this method too successful, Dresner (1947) produced large quantities of spores of the same fungus on a bush-bean medium. Spores were obtained at the rate of 10 per cent of the dry weight of the medium; this amounted to 60 grams per pound, or enough to cover about 3 acres.

Fig. 217. Close-up of applicator used for the distribution of spore dust, as in Fig. 216. (*Courtesy of S. S. Easter, U.S. Army, Engineer Corps.*)

One of the most carefully prepared bacterial spore-dust mixtures is that prepared for the artificial distribution of *Bacillus popilliae* Dutky, the cause of type A milky disease of the larva of the Japanese beetle (Dutky, 1942). The bacillus is cultivated in the bodies of Japanese-beetle grubs, which are crushed in a meat chopper, and the resulting spore suspension is standardized. This suspension is then added to the carrier, calcium carbonate, so that the mixture will contain a billion spores per gram of the dry material. The moist dust is then run through a mixing device and passed through a high-speed impeller-type blower to shear the agglomerated particles. Final drying is accomplished by drawing

heated air through the blower. This concentrated spore-dust suspension is then mixed with suitable quantities of dry carrier (talcum powder or marble flour) and stored until used. The final mixture contains 100 million spores per gram. The spore dust is usually applied by placing it in spots at intervals on the surface of the turf or soil, depending upon heavy dews or rains to wash the material into the soil. A hand corn planter of the rotary type, adjusted to deliver 2 grams of material per spot, may be used effectively. The spore dust may also be satisfactorily distributed by mixing it with a fertilizer and applying the two materials simultaneously.

CLIMATIC FACTORS AFFECTING MICROBIAL CONTROL

Without any question the success or failure of man's efforts to control insect pests by means of pathogenic microorganisms is closely related to climate and to the daily weather. Yet, in spite of the repeated emphasis given to the importance of these factors in applied insect pathology, only occasional or scant experimental attention has been given them by investigators in this field. A thorough comprehensive study of the fundamentals and general principles involved has yet to be made. Only after this has been accomplished will we be able to have a sound understanding of the microbial control of insects.

On the basis of our present knowledge the principal factors of climate, as they affect insect diseases, are temperature and humidity. Each of these is usually considered in terms of the other, and it is convenient for us to do so in the present discussion. It should be borne in mind that these factors also affect insects themselves to varying degrees at different times. As far as insect diseases are concerned, however, the effects of temperature and humidity upon the causative organism and on the defense mechanisms of the host are usually of prime importance.

All microbial agents, whether bacteria, fungi, protozoa, or viruses, have their minimum, optimum, and maximum ranges of temperature and humidity. Below a certain minimum temperature or above a certain maximum temperature the microorganism dies. Although many microorganisms flourish in a 100 per cent humidity, most of them are capable of withstanding only a certain amount of desiccation. There is always an optimum temperature and humidity at which the organism grows and develops best. The longer the optima are maintained the greater will be the over-all activity of the organism. Many of these minute forms of life are able to withstand wide variations in temperature and humidity by virtue of the spores or cysts that enclose their vital protoplasm.

In most instances the disease is likely to develop and spread the most rapidly at those temperatures and humidities which provide for the most luxuriant growth of the microorganism concerned. Most of the ento-

mogenous bacteria and fungi grow best at temperatures of between 25 and 30°C. There are exceptions, of course, and some microorganisms have optimum temperature requirements outside this temperature range. As a general rule, when the density of a susceptible insect population is high, when the average daily temperature falls within the range indicated above, when the relative humidity is high (90 per cent or above), and when the causative agent is present in adequate numbers, an outbreak of disease is extremely likely. The combination of a high temperature and a low humidity, or a low temperature and high humidity, may militate against widespread epizootics. The situation with regard to certain entomogenous fungi is a case at point.

It is generally agreed that the optimum conditions for the growth and development of entomogenous fungi include warm temperatures with concomitant high humidity. A situation such as exists in California, for example, with the wet season occurring during the cooler months and with dry to almost arid conditions prevailing during the warmer summer months, is at the outset an extremely unfavorable environment for the profuse development of fungi. The same applies to the other far western states, although more ideal conditions are approached in the northwestern parts of Oregon and Washington, where warm-season precipitation averages 15 to 30 inches from April to September, inclusive. During this same time of year, the average warm-season precipitation in California is between 2 and 5 inches except in the extreme northern part, where it averages 10 inches. This is not to say that in certain localized areas in California the conditions are not at least occasionally suitable for outbreaks of entomogenous fungi. Isolated natural outbreaks of fungous diseases of such insects as aphids, the cloverleaf weevil, thrips, and scale insects have been observed not infrequently.

Now compare these unfavorable climatic factors of California with the situation in Florida, where entomogenous fungi abound and where both natural and artificial control of insects has on occasions been obtained through the use of fungi. Florida is a low-lying subtropical peninsula, and the land is at no point more than 60 miles from sea water. The warm-season precipitation averages from 30 to 40 inches between April and September, inclusive. During Florida's rainy season the average temperature is between 70 and 80°F., while during California's rainy season the average temperature is between 40 and 50°F. The latter temperatures are unfortunately too low for the growth and complete development of most entomogenous fungi.

It is interesting to note that, whereas 75 species of entomogenous fungi on 150 insect host species have been reported from Florida, only 21 species on 25 hosts have been reported from California. It is recognized, of course,

that the fungi of Florida have perhaps been studied more thoroughly and for a longer time than have those of California, but the fact that fewer have been reported from the latter state is strongly indicative of the relative sparsity of fungi as compared with Florida.

Instances have been reported, however, in which disease outbreaks have occurred during periods of relatively low temperatures and high humidities. Billings and Glenn (1911), on occasions, found the chinch-bug fungus to be active under such circumstances. High humidity, however, was necessary, and these authors concluded that "moisture conditions have much to do with the appearance of chinch-bug disease in a field; artificial infection nothing." Müller-Kögler (1941) observed that whereas high relative humidities were conducive to infection of spruce sawfly larvae by *Spicaria farinosa* var. *verticilloides* Fron and *Beauveria bassiana* (Bals.), natural infection occurred readily at low temperatures (8°C.). Similar observations have been made in the case of certain bacterial and virus diseases in which outbreaks occurred during periods of comparatively low temperatures and high humidity. On the other hand, some outbreaks have been observed to occur in dry seasons with low humidities but high temperatures. As mentioned earlier, such situations are rather exceptional, and in the majority of cases the optimum conditions for disease outbreaks are concomitant high temperatures and high humidities.

In addition to Florida, many other tropical and subtropical regions of the world nurture the growth and development of entomogenous fungi because of the relatively high average temperature and the consistently high humidity and heavy rainfall. Examples have been reported from such places as Jamaica, Kenya, Trinidad, Ceylon, India, islands of the South Pacific, South America, and elsewhere. It has been observed in some instances that applications of fungi made during actual rains have been very successful. In South Africa, Skaife (1925) noticed that the grasshopper fungus, *Empusa grylli* (Fres.), developed profusely only in those localities which have a rainfall of over 14.5 inches during the 6-month period concerned. In some regions the incidence of entomogenous fungi is seasonal, appearing profusely during the spring or summer rains, and all but disappearing during the remainder of the year. The effect of the humidity may be very localized. In Jamaica, for example, the coffee scale (*Coccus viridis* Green) is more subject to attack by fungi on the lower slopes of the island than at high altitudes. Furthermore the fungus is more effective against those scales on the lower branches of the tree where the humidity is high than on the higher branches where the humidity is lower. With some insects the microclimate may be suitable for fungus growth even though the macroclimate is not. Thus the corn borer, inside

the ear of corn, and mealybugs down under the leaf sheaths are located where conditions of humidity are usually favorable.

Some, but not enough, attention has been paid to means of controlling the temperature and humidity in the field in such a manner as to enhance the effectiveness of entomogenous fungi. Humidity, of course, is much easier to control in the field than is temperature. For example, humidity might be raised by irrigation to such a point that fungous spores naturally present can germinate. Sprinklers or fog sprays used in the early morning or evening might be used over insect-infested fields. Since the drying effect of prevailing winds is not conducive to the luxuriant growth of fungi, this should be taken into account when determining the effectiveness of humidity control.

The relation of climatic factors to the outbreaks of bacterial diseases among insects does not appear to be so direct as it does in the case of fungous diseases. Epizootics caused by bacteria generally appear to be favored by warm humid weather. Actually very few pertinent observations have been made in this connection. Certain soil insects (*e.g.*, June-beetle larvae infected with *Micrococcus nigrofaciens* Northrup) have been seen to succumb to bacterial infections more readily in excessively wet soil than in drier soil. Temperature ranges appear to be less critical than do those of humidity. Thus there appears to be no significant difference in the incidence of milky disease in Japanese-beetle grubs at temperatures of 24 and 30°C. Wider ranges do modify the rate of progress of most bacterial infections.

The relation of climate to virus and protozoan infections has not been extensively studied. Although some workers (*e.g.*, Růžička, 1926) maintain that a damp cool atmosphere favors certain polyhedral virus diseases, most authorities believe that warm temperatures are more conducive to such outbreaks. All, however, are agreed that moisture is important. This can be seen in fields of alfalfa, for instance, where the incidence of disease in the alfalfa caterpillar increases following thorough irrigation. Some outbreaks of virus disease (*e.g.*, the polyhedrosis of the European sawfly) appear to have little relation to weather conditions other than extremes.

Although climate and weather are in themselves important factors, nevertheless throughout all these considerations one must be mindful of an equally important factor: density of population. As has been pointed out in Chap. 6, nearly all microbial diseases of insects are favored by high populations and host activity. Since warm temperatures and adequate relative humidities favor insect growth and development, as well as microbial life, it is understandable why the most spectacular outbreaks of disease among insects should be seen at times when the host

population, as well as the temperature and humidity, is high. In most instances, however, it should be remembered that the mortality caused by microbial diseases is not necessarily dependent upon density in exactly the same sense as it is with insect parasites and predators. To be sure, the spread of a disease is, to a considerable extent, dependent upon the density of the host population, but the initiation or appearance of the disease is largely dependent upon weather conditions, particularly the factors of temperature and humidity, probably more so than are the activities of insect parasites and predators. An infectious agent may be uniformly present in a population of high density, but unless weather conditions are favorable no appreciable epizootic may ensue.

Disadvantages of Microbial Control. Although some of the difficulties attending the widespread artificial use of microorganisms have already been discussed, it is well, at this point, to summarize the disadvantages, possible and real, of this method of biological control. All methods of insect control have their disadvantages, and the perfect method of controlling or eradicating insect pests is yet to be devised. The only intelligent approach to the evaluation of any method of control is through an honest acknowledgment of its disadvantages and shortcomings. Once recognized, these disadvantages may frequently be eliminated through adventuresome but sound research. Such may very likely be the case with microbial control methods.

Perhaps the greatest single disadvantage in the use of microorganisms in the control of insect pests has to do with the timing of the application in relation to environmental conditions. Wherever this difficulty has been largely solved or avoided, as in the case of type A milky disease of the Japanese beetle, control has been fairly or markedly successful. Conversely, instances in which this factor has not been surmounted, or where it has been ignored, have usually failed to accomplish effective control. This has been found to be a particularly important problem with the entomogenous fungi, most of which need to be applied at times of relatively high moisture and warmth. If microorganisms are distributed in fields during or just prior to dry, arid, or cool weather conditions, the likelihood of an effective "take" is remote. Much remains to be learned as to just what are the optimum times to apply the microorganisms.

Other disadvantages of microbial control include the necessity of maintaining the vitality and virulence of the infecting agent, especially of those microorganisms not possessing a cyst or spore stage; the possibility that resistant populations will develop after prolonged use of the microorganisms; and the possibility that farmers and growers will rely too heavily upon microbial methods, neglecting such complementary measures as the use of insecticides and parasitic insects.

The effect that heavily distributed entomogenous microorganisms may have upon plants and higher animals needs always to be considered. There appears to be little likelihood, however, that microorganisms naturally pathogenic to insects would cause serious injury to other animals or to plants. No authenticated case of such a thing happening has yet been reported. As was brought out earlier in this book, most of the bacteria pathogenic for higher animals have little or no virulence for insects, and microorganisms naturally pathogenic for insects, as a rule, have not been found pathogenic for vertebrates under experimental conditions.

Of considerable importance is the effect that microorganisms pathogenic for a given insect pest may have upon the insect parasites and predators of that pest. Only a few observations have been made in this regard, but enough has been learned to suggest that close attention must be paid to this relationship whenever the artificial dissemination of microorganisms is contemplated. Sometimes the insect parasites and the diseases work in a complementary or supplementary fashion. This has been observed, to some extent, in alfalfa fields infested with caterpillars of the alfalfa butterfly (*Colias*). In fields where the polyhedral "wilt" disease is present but not abundant among the caterpillars, the smaller larvae may be parasitized by *Apanteles* while the larger larvae may be killed by the virus. In fields where the disease is destroying large numbers of caterpillars, the number parasitized by *Apanteles* is often less than it is in adjacent fields. In *Wipfelkrankheit* of the nun-moth caterpillar a high incidence of disease in heavily infested areas has been reported to cause many of the tachinid parasites to migrate to areas of lighter infestation. Instances have been observed in which the insect parasites have dominated the situation after the disease had first reduced the number of hosts to a controlled level. Thus, in nature, the occurrence of disease may work in harmony with other host-parasite balances. Undoubtedly instances occur in which parasitized insects are killed by disease before the parasite can complete its development. In such cases it must be determined which of the two biological agents has the greater control value, and then this agent should be favored over the other. As in most phases of biological control, so it is that the introduction of pathogenic microbes should be accomplished in such a manner as not to upset any balance of nature that is operating in man's favor.

Merely because a pathogenic microorganism is capable of decimating certain populations of insects is no assurance that the over-all effect will be beneficial. A disease may be initiated or may suddenly appear in a population that has attained a state of equilibrium, and seriously disturb the existing natural balance. What we have reference to might best be explained by describing the observations made by Ullyett and Schonken

(1940) on the effect of the fungus *Entomophthora sphaerosperma* Fres. on a population of *Plutella maculipennis* Curt. in South Africa. According to these workers the mode of action of the fungus on the host population under their observation is essentially similar to that of an insecticide which, although it may produce excellent immediate results, may allow the insect to return ultimately to its original or to an even greater popula-

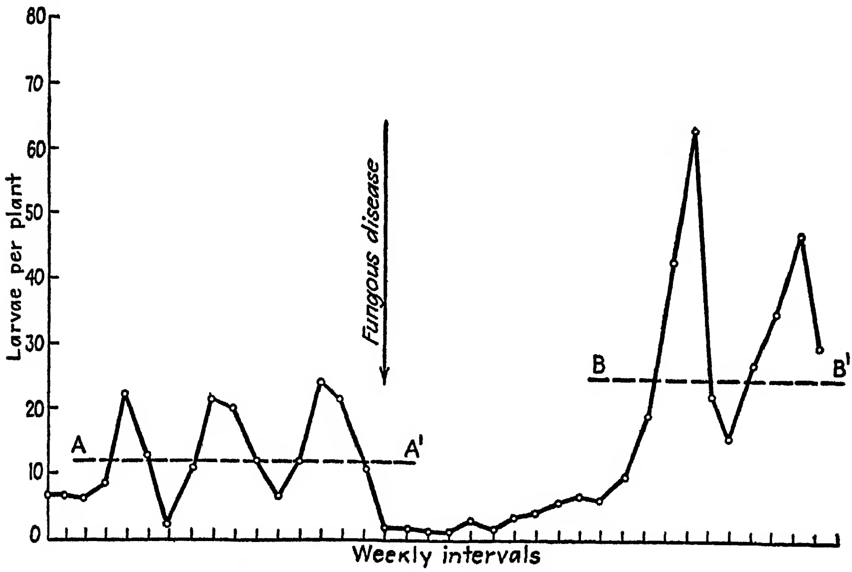

Fig. 218. Curve showing the progress of a population of *Plutella maculipennis* Curt., with an intervening epizootic of *Entomophthora sphaerosperma* Fres. See text for further explanation. (*After Ullyett and Schonken, 1940.*)

tion level. The progress of a *Plutella* population with an intervening attack of disease was followed, as depicted in Fig. 218. In the beginning the population exhibited normal fluctuations of the type found in equilibrium systems and possessed an average density AA^1 that was low enough to exclude economic damage to the crop at the peaks of the curve. The sudden appearance of the disease was followed by a rapid decline in the number of host individuals, and a low population was maintained throughout the ensuing period of favorable weather. With the disappearance of the disease, Ullyett and Schonken noticed that the host population quickly recovered and that, since the biotic factors (insect parasites and predators) normally responsible for control had been largely destroyed during the epizootic, it was able to build itself up to much greater proportions than had previously been the case. A new series of fluctuations then became evident, and these were taking place about a new average

density represented by the line BB^1. This was approximately twice the value of the previous mean AA^1, and economic damage to the crop occurred at or near the peak periods. Thus it would appear that the effects of a disease outbreak may, under certain conditions, be ultimately disadvantageous, economically speaking. Even in such cases, however, the repeated artificial introduction of the disease agents may be effective in preventing any significant increase in the average density of the population.

Advantages of Microbial Control. The principal advantages of using biological methods of controlling insects are rather obvious and do not require an extensive explanation. Most of the advantages that attend the use of insect parasites and predators also apply to the use of microorganisms.

In the first place, successful microbial control is a relatively inexpensive method of reducing populations of destructive insects. Large quantities of most microorganisms can be cultivated and produced inexpensively and in a relatively short period of time with only a small outlay of media and cultivating apparatus. In addition, they can be easily distributed as sprays and dusts.

Microbial control is a natural method of control, and as such it may increase its effectiveness naturally after once being introduced into an area. If conditions are optimum, the introduced microorganisms may spread of their own accord, resulting in widespread epizootics. Furthermore, as in the case of many insect parasites, once an infectious organism is introduced into an area, it may maintain itself more or less permanently for the entire season or even for several years, as has been demonstrated in the case of type A milky disease of the Japanese beetle.

Not the least of the advantages of microbial control is the fact that most entomogenous microorganisms are essentially harmless to animals and plants and may be applied in heavy doses without damaging these forms of life. The safety to human health is an advantage that biological control has over the heavy deposits of many insecticides. The dangers and worries attending the effect of chemical residues upon the host plants or upon the consumers of the plants ordinarily is not a factor in microbial control.

An advantage that microbial control might have over the use of parasitic insects concerns the fact that microoganisms generally are not appreciably affected by insecticides. In other words, the concomitant use of insecticides would not be likely to interfere with the pathogenic action of the entomogenous microoganism.

Just as the presence of disease in an insect population may work to the disadvantage of the insect parasites of that host, so also may the presence of disease cause an actual increase in the proportion of parasites to the

host insects. Such a state of affairs is indicated by the data obtained by King and Atkinson (1928) during their studies on the biological control of the red-backed cutworm, *Euxoa ochrogaster* (Guen.), in Saskatchewan. As shown in Fig. 219, in one area studied, the mortality from disease rose from 13 per cent on May 17 to 73 per cent on June 25. During the same period, the moth emergence fell from 69.8 per cent to 1 per cent. Now when a comparison is made of the parasitism (by *Meteorus vulgaris* Cress. and other species) curve with the other two, an important fact is revealed. During the period of June 15 to 25 the percentage of parasite emergence increased from 23 to 26, while the percentage of moth emergence decreased from 13 to 1. (The estimated effective parasitism for the year was only 61 per cent.) Therefore, King and Atkinson conclude that the disease not only was of great effect in destroying most of the larvae present during the year 1925 but also was effective in greatly increasing the proportion of parasites to moths emerging, thus favoring the possibility of high parasitism the following year.

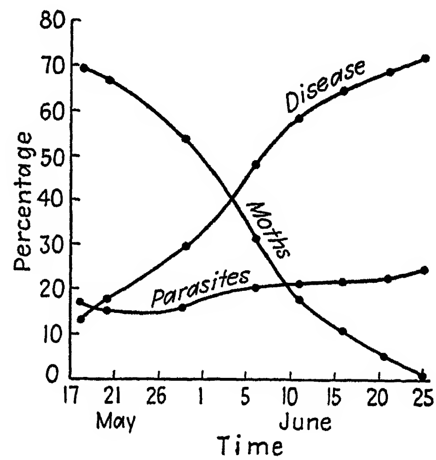

Fig. 219. Graphic representation of the mortality of *Euxoa ochrogaster* (Guen.) from insect parasitism and disease as studied in Saskatoon, Canada, by King and Atkinson (1928). (For explanation see text.)

The Future of Microbial Control and Insect Pathology. To predict the nature of future developments in any branch of science is a precarious undertaking; this is especially true in the case of applied insect pathology. It is of value, however, for every scientist to take occasional glimpses into the future if for no other reason than to assist him in maintaining the proper perspective of his current work in relation to the potentialities of his science as a whole. Although insect pathology itself may never command as much attention as, for example, the fields of plant pathology or vertebrate pathology, it may be the forerunner of a field of endeavor that might logically be designated as "invertebrate pathology." In such a situation insect pathology would be not so much a branch of entomology as the backbone of invertebrate pathology. The microbial control aspects of insect pathology as well as those of the remainder of invertebrate pathology would fall, as they do now, into the broad realm of applied ecology.

The use of microorganisms in the control of insect pests appears to be just emerging from the metaphysical stage. Not only has the subject

been a neglected one, but for many years it has been a sort of no man's land between entomology and microbiology. A great deal of the early work, particularly that with entomogenous fungi, was accomplished by scientists such as plant pathologists who had to acquire most of their knowledge of insects and insect populations from firsthand experience alone. It has been only recently that the field of insect pathology has come into its own, with its own specially trained men. In this sense, the field of microbial control has nearly all its "future" still before it.

The relative novelty of the idea of using disease-producing microorganisms to control insects, as is the case with the sinister biological warfare among human beings, makes good newspaper copy and radio description. Unfortunately this type of popular publicity, when unrestrained, usually does not work for the good of science. Hopes are built up among farmers, growers, and other agriculturists, on the basis of preliminary work that as frequently as not shows the impracticability of the microbial-control method in that particular instance. Such dashed hopes are damaging to the progress of the research, which must depend upon public financial and moral support. The past has been characterized by too much of this sort of thing—let us hope that the future will not be, even though the particular scientists concerned are often not responsible for the premature and unscientific publicity they get. Only after the details of any particular microbial-control procedure have been worked out and its potentialities accurately determined should the insect pathologist be obligated to make the results of his work known to the public at large.

Another aspect that needs to be curbed in the future is the mistaken idea that microbial control, or for that matter biological control generally, necessarily operates in direct opposition to chemical-control methods. The common objective of both lines of work should be that of the most efficient and effective control of insect pests from the standpoint of man's welfare and economic benefit—regardless of whether in any particular case it is microbial control or chemical control that does the job. Actually it appears that the two may often work hand in hand to the best advantage. Thus, as is done in the case of type A milky disease of the Japanese beetle, chemical insecticides may often be applied to obtain immediate control, while microorganisms are used for projected or long-range control.

Microbial control should therefore be considered as an integral part of man's effort to control the insects that plague him and his crops. Without much doubt, such a biological method, because of the difficulties attending its use, requires more painstaking and laborious study in order to learn how to use it than do most methods of control—but once mastered the rewards are also probably greater. Because of the complexities con-

nected with microbial control, numerous workers have been quick to condemn its use generally. Such a pessimistic attitude is as unscientific as is that which makes overenthusiastic claims for the method. As far as future expectations are concerned, the truth appears to lie somewhere between these two extremes.

No one should envision microbial control as the panacea to our insect problems. It is safe to say that for many insects microbial control would almost never be possible, let alone being practical. For many additional insects, chemical control would be almost as inexpensive and as effective as microbial control. Then there are pests that will probably always be effectively controlled by insect parasites and predators. Nevertheless this leaves a large reservoir of insect pests that might be effectively controlled by microbial methods if the proper procedures could be worked out and all the contributing factors understood. Even in these cases, however, we should look for ways in which the microbial method might be combined with the use of chemicals, insect parasites, or predators.

Very much to the point is Petch's (1921) declaration that "the problem which has yet to be solved by those who wish to control insects by means of fungi is how to create an epidemic at a time when such an epidemic would not occur naturally." A similar statement might be made concerning bacterial, virus, and protozoan diseases of insects as well. From the control standpoint, it is the factors that govern the incidence of these diseases that need further investigation, even more so than do the diseases themselves. Once these factors are elucidated it may then be possible to utilize them in the control of insect pests. This does not mean that we should assume an overly pessimistic attitude. Even at the present time we are entitled to express the hope that in a few worth-while instances the artificial dissemination of disease organisms under the proper conditions may aid in at least the partial or seasonal reduction of certain insect pests. Furthermore we are probably justified in believing, in some cases at least, that on the one hand repeated introductions of disease organisms into a susceptible insect population may keep that population below an economically destructive level much the same as does the repeated introduction of parasitic insects, and that on the other hand once established in an area certain diseases may maintain themselves and take a small but significant toll of insects year after year.

The important thing, however, is that we get down to fundamentals before going pell-mell ahead attempting to control insects before we really know what we are doing. This still requires a tremendous amount of the type of research usually characterized as "fundamental" or "basic." We must be willing to spend the time, money, and energy necessary to accomplish this research. If we are not willing to do this, then we cannot

expect to derive very much practical usefulness from our efforts. Also needed is the sympathetic, moral, and financial support of basic research in all the various biological relationships existing between insects and microorganisms. Up to now, with a few noteworthy exceptions, the mistake has too often been made of jumping ahead to obtain as many of the practical benefits from the field of insect pathology as possible without paying for them in terms of good sound research. The few initial investigations have for some time been yielding diminishing returns. We must now go back and build a scientifically firm foundation of fundamental knowledge to place under the rather flimsy structure now existing. Having this foundation we can then repair the defective structure and build anew until we know for certain whether or not microbial control can be of practical usefulness to man in his efforts to control insect pests.

It would be a serious mistake to close this volume with any implication that microbial control was the only practical consequence to be hoped for from insect pathology. It is even questionable whether microbial control is the most important of the various aspects of insect pathology. As was brought out in the beginning pages of this book, insect pathology has in the past and will in the future contribute heavily to most phases of entomology and to other branches of biology as well. But even without this realization of immediate practical usefulness, insect pathology is worth all the time, effort, and money we can put into it, if only for the purpose of better understanding insect life in general. The fully trained insect pathologist really learns to know insects as well as the microoganisms that infect them. It is a field in which the scientist may exercise his capacity of ingenious experimentation to the fullest. It is also a field in which he can find real happiness and which he can thoroughly enjoy because of its relative lack of boring routine. The student who goes into the field of insect pathology for the sole purpose of making practical use of it will not find the contentment found by him who goes into the field because he is compelled to do so by virtue of his deep interest or insatiable curiosity in this area of Nature's activity.

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